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TESIS DOCTORAL

Caracterización genética y funcional de la bacteria probiótica "Pediococcus parvulus" 2.6

Genetic and functional characterization of the probiotic bacterium "Pediococcus parvulus" 2.6

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PRESENTADA POR

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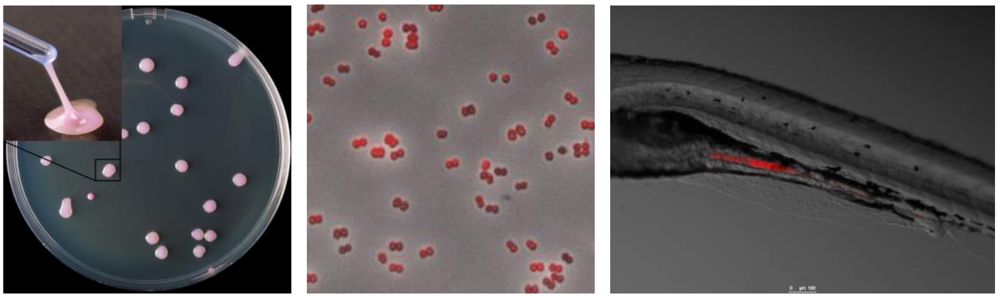
CARACTERIZACIÓN GENÉTICA Y FUNCIONAL DE LA BACTERIA PROBIÓTICA *PEDIOCOCCUS PARVULUS* 2.6



GENETIC AND FUNCTIONAL CHARACTERIZATION OF THE PROBIOTIC BACTERIUM *PEDIOCOCCUS PARVULUS* 2.6

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La Dra. Paloma López García y la Dra. Mª Luz Mohedano Bonillo, del Centro de Investigaciones Biológicas (CIB) adscrito al Consejo Superior de Investigaciones Científicas (CSIC),

Certifican:

Que la presente memoria titulada: “Caracterización genética y funcional de la bacteria probiótica *Pediococcus parvulus* 2.6” ha sido realizada por el doctorando D. Adrián Pérez Ramos, bajo su dirección en el laboratorio de Biología Molecular de Bacterias Gram-positivas del CIB, y autorizan su presentación para la defensa de tesis y obtar al grado de Doctor por la Universidad Complutense de Madrid.

Y para que así conste a los efectos oportunos, firman la presente en Madrid en febrero de 2019.

Fdo.: Dra. Paloma López García

Fdo.: Dra. Mª Luz Mohedano Bonillo

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Index

Abbreviations

2.6p: *Pediococcus parvulus* 2.6[pRCR12]

2.6NRp: *Pediococcus parvulus* 2.6NR[pRCR12]

CCR: carbon catabolite repression

CcpA: catabolite control protein A

cre: catabolite repression element

Cys: cysteine

DC: Dendritic cell

DNA: deoxyribonucleic acid

EFSA: European Food Safety Authority

EPS: exopolysaccharide

FAO: Food and Agriculture Organization of the United Nations

FBP: fructose-1,6-bisphosphate

FDA: Food and Drug Administration from the United States

FOS: fructooligosaccharide

GFP: green fluorescent protein

GIT: gastrointestinal tract

GOS: galactooligosaccharide

GRAS: generally Recognized as Safe

HePS: heteropolysaccharide

His: histidine

HoPS: homopolysaccharide

HprK/P: HPr kinase/phosphorylase

IL8: interleukin 8

IL10: interleukin 10

kbp: kilo-base pairs

LAB: lactic acid bacteria

mCherry: synthetic monomeric variant of the red fluorescent protein

MyD88: myeloid differentiation primary response gene 88

NF-kB: nuclear factor kappa-light-chain-enhancer of activated B cells

PAMP: pathogen-associated molecular pattern

PEP: phosphoenolpyruvate

PRR: pattern recognition receptor

PTS: phosphoenolpyruvate:sugar phosphotransferase system

PTS^{gut}: PTS for sorbitol utilization

PYR: pyruvate

QPS: qualified Presumption of Safety

SCFA: short-chain fatty Acid

TLR: Toll-like receptor

TNFα: tumor necrosis factor alpha

WHO: World Health Organization of the United Nations

Resumen

Los β-glucanos lineales y ramificados, especialmente aquellos unidos mediante enlaces β-(1,3) y sintetizados por plantas y hongos, pueden actuar como inmunomoduladores ejerciendo efectos beneficiosos sobre la salud humana. Estos homopolisacáridos (HoPS) se han utilizado extensamente en la medicina tradicional de ciertas culturas, y en nuestros días son considerados como compuestos bioactivos para el desarrollo de nuevos alimentos funcionales. Así también, los β-glucanos se utilizan como sustitutivos a los tratamientos antibióticos en industrias como la acuicultura y la ganadería.

Se ha descrito un (1,3)-β-glucano ramificado en posición *O*-2 producido principalmente por las bacterias del ácido láctico (BAL), entre las cuales *Pediococcus parvulus* 2.6 es el prototipo de bacteria productora de este β-glucano. En este HoPS, se han investigado sus características físico-químicas, su producción y su influencia en el metabolismo bacteriano. Además, alguna de las propiedades probióticas de la bacteria como su resistencia al estrés gastrointestinal o su adhesión a células epiteliales han sido caracterizadas *in vitro*, así como la capacidad de su β-glucano de modular macrófagos humanos.

En esta tesis, se ha descrito un método inmunológico capaz de detectar y cuantificar específicamente el β-glucano bacteriano (Capítulo 1). Esta técnica se basa en el reconocimiento de este β-glucano por un anticuerpo específico para *Streptococcus pneumoniae* serotipo 37. Este neumococo produce una capsula que se compone por un β-glucano muy similar al producido por BAL. El método se ha utilizado para la caracterización de la actividad enzimática de la glicosiltransferasa GTF, responsable de la producción del β-glucano en *P. parvulus* 2.6, mediante el uso de vesículas membranosas que contenían el enzima determinando las constantes catalíticas (Capítulo 1). Por otra parte, este método fue validado para la cuantificación del HoPS en diferentes estados: (i) puro (Capítulo 1), (ii) contenido en sobrenadantes de cultivos bacterianos (Capítulos 1, 4, 5 y 7), (iii) unido a las bacterias productoras (Capítulo 5) y (iv) contenido en matrices alimentarias (Capítulo 6).

Se ha descrito el borrador del genoma del *P. parvulus* 2.6 (Capítulo 2), siendo este el primer genoma secuenciado de esta especie. Su tamaño se ha estimado en 2.236 kpb y se han detectado 2.241 genes. El análisis del genoma reveló la ausencia de determinantes genéticos involucrados en actividades indeseadas como puede ser la resistencia a antibióticos o la producción de aminas biógenas. También, se ha descrito la presencia de la agrupación genética *gutFRMCBA* (operón *gut*), cuyos genes están

implicados en la utilización del sorbitol por la bacteria. El operón se compone de 6 genes: el gen *gutF* que codifica una sorbitol-6-P deshidrogenasa; los genes *gutR* y *gutM* que codifican dos posibles reguladores; y los genes *gutC*, *gutB* y *gutA* que codifican el dominio EII de un sistema de transporte fosfotransferasa dependiente de fosfoenolpiruvato específico para el sorbitol.

Por lo tanto, la utilización del sorbitol como fuente de carbono para el metabolismo de *P. parvulus* fue investigada (Capítulo 4). Primero, se estandarizaron las condiciones óptimas para su metabolismo (Capítulo 7). El cometabolismo de la glucosa y el sorbitol mostró que la cepa 2.6 metaboliza primero la glucosa y después el sorbitol, indicando que la expresión del operón *gut* se encuentra sujeta a represión por catabolito en el crecimiento en presencia de glucosa. El análisis de los flujos metabólicos y la producción del sorbitol en *P. parvulus* 2.6 han demostrado que el sorbitol puede actuar como precursor para la producción del β-glucano (Capítulo 4).

Por otra parte, se ha desarrollado un vector para evaluar promotores que permite realizar análisis transcripcionales en BAL, basado en la expresión del gen *mrfp* que codifica la proteína fluorescente roja mCherry (Capítulo 3). Nos hemos valido de este vector para investigar la regulación transcripcional de la expresión del operón *gut* pediocócico de utilización del sorbitol (Capítulos 4 y 7). Para la realización de este estudio, la posible secuencia promotora (P_{gut}) así como los dos genes que codifican dos posibles proteínas reguladoras (GutR y GutM), fueron clonados, de forma independiente o en conjunto, produciendo fusiones transcripcionales con el gen *mrfp*. El estudio se llevó a cabo en dos huéspedes heterólogos, *Lactobacillus plantarum* y *Lactobacillus casei*, que también utilizan el sorbitol. Los resultados mostraron que en el crecimiento en presencia de sorbitol se produjo una *trans*-complementación en la activación de P_{gut} en *L. plantarum*, pero no así en *L. casei*. Los resultados también indicaron que GutR es un activador transcripcional y que GutM es necesaria para el correcto funcionamiento de P_{gut}. Además, se ha postulado un modelo para la regulación de la expresión de *gutFRMCBA* en *P. parvulus* 2.6.

Se ha evaluado *in vitro* la respuesta de *P. parvulus* 2.6 y de su cepa isogénica 2.6NR a varios estreses tecnológicos (Capítulo 7). Los resultados mostraron un efecto protector del β-glucano frente a un estrés alcohólico, y además, el etanol puede ser un posible inductor de la producción de EPS.

Las cepas de *P. parvulus* 2.6 fueron marcadas fluorescentemente mediante la transferencia del plásmido pRCR12, el cual porta el promotor neumocócico fuerte P_x fusionado al gen *mrfp* (Capítulo 5). Hasta donde nosotros sabemos, esta es la primera vez que se marca fluorescentemente *P. parvulus*. Además, la producción constitutiva de la proteína mCherry a partir de pRCR12 no afectó ni al crecimiento de las cepas ni a la producción del β-glucano por la cepa 2.6. También, se evaluó la capacidad de adhesión a enterocitos de 2.6[pCRC12] (2.6p) y 2.6NR[pRCR12] (2.6NRp) utilizando un modelo *in vitro* de células Caco-2. Los resultados revelaron que la presencia del β-glucano unido a la superficie de 2.6p aumenta la capacidad de unión de la bacteria, como ya se detectó previamente en la cepa 2.6. Además, la adhesión de 2.6NRp fue mejorada mediante la suplementación con el β-glucano purificado, demostrando así el efecto positivo del β-glucano en la interacción bacteria-enterocito.

Las propiedades probióticas de *P. parvulus*, así como la capacidad inmunomoduladora de su β-glucano han sido investigadas usando modelos *in vivo* de pez cebra (Capítulo 5). Para el estudio de la colonización de las cepas marcadas fluorescentemente de *P. parvulus* se utilizó un modelo larvario gnotobiótico. La señal fluorescente roja emitida por las bacterias en el interior del tracto gastrointestinal (TGI) de las larvas fue detectada, lo que permitió monitorizar en tiempo real la colonización bacteriana. El análisis de las bacterias viables en el interior de las larvas reveló que la colonización del TGI del pez cebra por la cepa 2.6p fue mejor que por la cepa 2.6NRp, indicando un efecto positivo del β-glucano en la colonización. Además, las larvas gnotobióticas, pretratadas con las soluciones bacterianas, fueron infectadas con el patógeno *Vibrio anguillarum*. Ambas cepas fueron capaces de contrarrestar los efectos de la infección, reduciendo la mortalidad de los peces cebra, sin embargo la cepa 2.6p lo hizo de una manera más eficaz, poniendo en valor su potencial probiótico. Por otra parte, el modelo gnotobiótico del pez cebra fue utilizado para estudiar la modulación del sistema inmune innato del pez cebra por parte del β-glucano de *P. parvulus* 2.6 (Capítulo 5). El tratamiento con el β-glucano purificado provocó una reducción en la expresión genética de la IL8, TNFα y MyD88 en las células inmunes del pez cebra, lo que sugiere una respuesta antiinflamatoria. Posteriormente, la línea transgénica de pez cebra *Tg*(*mpx: GFP*) fue utilizada para desarrollar un modelo de inflamación inducida, mediante el seccionamiento de la región apical de la cola. Las larvas de esta línea transgénica expresan la proteína GFP de forma constitutiva en los neutrófilos, así se puede detectar su reclutamiento a la zona de inflamación y su proliferación mediante medidas de fluorescencia. La exposición de las larvas al β-glucano bacteriano provocó una reducción significativa del reclutamiento y proliferación de los neutrófilos. Estos resultados mostraron el potencial del β-glucano pediocócico como un agente antiinflamatorio.

Finalmente, se investigó la capacidad de las cepas de *P. parvulus* para fermentar matrices alimentarias basadas en avena, cebada y arroz (Capítulo 6). *P. parvulus* no fue capaz de fermentar la matriz de cebada, sin embargo fermentó las matrices de arroz y avena, esta última más eficazmente. Las fermentaciones tanto con 2.6 como con 2.6NR permitieron observar como la producción del β-glucano por la primera cepa resultó en un incremento de la viscosidad de las matrices. Además, el potencial prebiótico de estos alimentos generados, fortificados con la producción *in situ* del β-glucano bacteriano, ha sido validado con la cepa *L. plantarum* WCFS1. Tanto el crecimiento de WCFS1 como su supervivencia a condiciones de estrés del TGI fueron superiores cuando la bacteria fue incluida en los alimentos biofortificados con el β-glucano.

Summary

Linear and branched β-glucans, particularly those linked by β-(1, 3) bounds synthesised by plants and fungi, can act as immunomodulators and therefore can be beneficial to human health. These homopolysaccharides (HoPS) have been extensively used in traditional medicine, and now are considered to be potentially useful for the development of functional foods. Also, β-glucans have started to be used in the livestock and aquaculture industries as substitutes for antibiotics to prevent infection.

A 2-substituted (1,3)-β-glucan produced primarily by lactic acid bacteria (LAB) has been described, in particular by *Pediococcus parvulus* 2.6, which is the architype of bacteria producing this β-glucan. For this reason, the physico-chemical characteristics of this HoPS from the 2.6 strain, its production and influence on metabolism have been previously investigated. Moreover, certain probiotic properties of the 2.6 strain such as resistance to gastrointestinal stress or adhesion to epithelial cells, as well as the modulating effect of its β-glucan on human macrophages, have been characterized *in vitro*.

In this thesis, an immunological method for the specific detection and quantification of the 2-substituted (1, 3)-β-glucan is described (Chapter 1). This technique is based on the recognition of the bacterial β-glucan by a specific antibody against *Streptococcus pneumoniae* serotype 37. This pneumococcal strain produces a capsule composed of a similar β-glucan to the LAB producers. This method has been used for the characterization of the enzymatic activity of the GFT glycosyltransferase responsible for the β-glucan production in *P. parvulus* 2.6 (Chapter 1). The study was performed in membrane vesicles containing the GTF protein, and the catalytic constants were determined. In addition, this method was validated for quantification of this HoPS in different states: (i) pure (Chapter 1), (ii) included in bacterial cultures supernatants (Chapters 1, 4, 5 and 7), (iii) attached to the producing bacteria (Chapter 5) and (iv) included in food matrices (Chapter 6).

The draft genome of *P. parvulus* 2.6 has been described (Chapter 2), this being the first genome sequenced for this species. Its size was estimated as 2.236 kbp, containing 2.241 genes. Its analysis revealed the absence of genetic determinants for unwanted activities such as antibiotic resistance or biogenic amine production. It also showed the presence of the *gutFRMCBA* (*gut*) operon involved in sorbitol utilisation. This operon is composed of six genes: *gutF* encodes a sorbitol-6-P dehydrogenase; *gutR* and *gutM*

encode two putative regulators; and *gutC, gutB* and *gutA* encode the EII domain of a phosphoenolpyruvate:sorbitol phosphotransferase transport system.

Therefore, the utilization of sorbitol as a carbon source by *P. parvulus* was investigated (Chapter 4). The conditions for the best sorbitol metabolism were standardised (Chapter 7). A co-metabolism with glucose and sorbitol showed that the 2.6 strain first metabolises the glucose and then the sorbitol, indicating that the expression of the *gut* operon is subject to catabolite repression upon growth in the presence of glucose. Analysis of the metabolic fluxes and the β-glucan production in *P. parvulus* 2.6 has demonstrated that sorbitol can act as a precursor for the production of the β-glucan (Chapter 4).

Furthermore, a promoter-probe vector was developed to perform transcriptional analyses in LAB, based on the expression of the *mrfp* gene which encodes the red fluorescent protein mCherry (Chapter 3). This vector was used to investigate the transcriptional regulation of the expression of the pediococal sorbitol utilisation *gut* operon (Chapters 4 and 7). For this study, the putative promoter sequence (P_{gut}) and two genes encoding two putative regulatory proteins (GutR and GutM) were cloned independently, or together, in transcriptional fusions with the *mrfp* gene. The study was carried out in two heterologous hosts, *Lacobacillus plantarum* and *Lactobacillus casei*, which also utilise sorbitol. The results demonstrated *trans*-complementation for activation of P_{gut} in *L. plantarum* but not in *L. casei*, upon growth in the presence of sorbitol. The results indicated that GutR is a transcriptional activator and GutM is needed for the correct functionality of P_{gut}. In addition, a model for regulation of expression of the *gutFRMCBA* in *P. parvulus* 2.6 has been postulated.

Responses of *P. parvulus* 2.6 and its isogenic β-glucan non-producing 2.6NR strains to various *in vitro* technological stresses have been evaluated (Chapter 7). The results showed a protective effect of the β-glucan to alcoholic stress and, also that ethanol could be an inducer of the EPS production.

P. parvulus strains were fluorescently labelled by transferring the pRCR12 plasmid which carries the strong pneumococcal P_x promoter fused to the *mrfp* gene (Chapter 5). As far as we know this is the first fluorescent labelling of *P. parvulus*. In addition, the constitutive production of mCherry from pRCR12 did not affect growth of the pediococcal strains nor the β-glucan production by 2.6. Also, bacterial adhesion of

2.6[pRCR12] (2.6p) and 2.6NR[pRCR12] (2.6NRp) to enterocytes was assessed in an *in vitro* Caco-2 cells model. The results revealed that the presence of the β-glucan attached to the 2.6p surface enhances the bacterial binding capacity as previously detected for the 2.6 strain. Also, the adhesion of 2.6NRp was improved by supplementation with the purified β-glucan, demonstrating the positive effect of the β-glucan in the bacterium-enterocyte interaction.

The probiotic properties of *P. parvulus*, as well as the immunomodulatory capacity of its β-glucan, have been investigated by using *in vivo* zebrafish models (Chapter 5). A gnotobiotic larval model was used to study the colonising ability of fluorescent-labelled *P. parvulus* strains. The red fluorescent signal emitted by the bacteria was detected inside the gastrointestinal tract (GIT) of the larvae, thereby monitoring the bacterial colonisation in real time. Analysis of viable bacteria inside the larvae revealed that the 2.6p strain colonises the zebrafish GIT better than 2.6NRp, indicating a positive effect of the β-glucan in colonisation. Furthermore, the gnotobiotic larvae, pre-treated with the bacterial solutions, were infected with the pathogen *Vibrio anguillarum*. Both strains were able to counteract the infection, reducing the zebrafish mortality, but strain 2.6p in a more efficient manner, supporting the probiotic potential of the strain. In addition, a gnotobiotic zebrafish model was used to study the modulation of the innate immune system of zebrafish by the *P. parvulus* 2.6 β-glucan (Chapter 5). The treatment with the purified β-glucan provoked a reduction in the gene expression of IL8, TNFα and MyD88 in zebrafish immune cells, suggesting an anti-inflammatory response. Subsequently, the zebrafish *Tg*(*mpx: GFP*) line was used to perform an induced inflammation model, by cutting the tail´s apical region off. This transgenic line expresses the GFP protein constitutively in the neutrophils, so their recruitment to the inflammation zone and their proliferation can be detected and quantified by fluorescent measurements. The exposure to the bacterial β-glucan provoked, in the treated larvae, a significant reduction of neutrophilic recruitment and proliferation. These results showed the potential of the pediococcal β-glucan as an anti-inflammatory agent.

Finally, the ability of *P. parvulus* strains to ferment oat, barley and rice matrices was investigated (Chapter 6). *P. parvulus* was not able to ferment the barley matrix, and fermented the oat matrix better than the rice one. Fermentations with both 2.6 and 2.6NR strains showed that the β-glucan production by the former results in an increase of viscosity of the matrices. In addition, the prebiotic potential of the fermented cereal-based foods fortified with the β-glucan produced *in situ* by *P. parvulus* 2.6 has been validated with *L. plantarum* WCFS1 strain. The growth and survival under GIT condition of the WCFS1 strain was superior when the bacterium was included in the bacterial β-glucan enriched foods.

Introduction

1. Functional food

The denomination of functional food refers to those foods that, in addition to having nutritional value, contain a biologically active component that confers some benefit to the health of the consumer. Thus, the consumption of these foods could lead to the improvement of certain physiological functions, and/or to the prevention or the reduction of risk of suffering a disease. Biologically active components may be living microorganisms such as bacteria or yeasts that are called probiotics; or they may be indigestible carbohydrates called prebiotics. Many of these foods are fermented foods, where probiotics can be part of these fermentation processes. Thus, in ancient times the human beings transformed milk into fermented dairy products through the use of bacteria and yeasts, without knowing the scientific basis of the processes. These dairy products were easily transported, stored and preserved for long periods of time. In addition, their healthy properties were known, as they were used to treat certain diseases (Ozen and Dinleyici, 2015). Therefore, yogurt and other fermented milks can be considered as the first functional foods.

1.1. Probiotics

The term ‘probiotic’ literally means ‘for life’, and comes from the combination of the Latin word ‘pro’ and the Greek word ‘bios’. It was introduced in 1953 by the German scientist Werner Kollath to designate ‘active substances essential for a healthy development of life’; and in 1965, this term was used by Lilly and Stillwell to represent ‘substances secreted by one organism which stimulate the growth of another’ (Gasbarrini et al., 2016). Already at the beginning of the 20th century, Elie Metchnikoff associated the improved longevity of Bulgarian people with the regular consumption of yogurt. He proposed that the Bulgarian bacillus (discovered by Stamen Grigorov) responsible for yoghurt fermentation counteracted the rotting effects of gastrointestinal metabolism, which contributes to disease and aging (Gasbarrini et al., 2016).

Currently, the most accepted definition is that offered by a consensus of the Food and Agriculture Organization (FAO) and the World Health Organization (WHO), which described probiotics as ‘living microorganisms which when administered in sufficient quantities confer a benefit to the health of the host’ (FAO/WHO, 2002).

1.1.1. Criteria for probiotic selection

Fuller (1989) listed all the characteristics that a probiotic must possess, and they were: (i) It should exert a beneficial effect on the animal host. (ii) It should be a non-pathogenic and non-toxic microorganism. (iii) It should remain in the form of viable cells, preferably in large quantities. (iv) It should be able to survive and have metabolic activities in the intestinal environment, e.g. must be resistant to low pH and organic acids. (v) It should be stable and able to remain viable for long periods under storage and field conditions.

Probiotics, being living organisms and being used in human food and animal feed, must satisfy certain safety requirements, and thus must have GRAS (Generally Recognized As Safe) status by the Food and Drug Administration (FDA) in the United States, or QPS (Qualified Presumption of Safety) status by the European Food Safety Authority (EFSA) in Europe. Historically, lactobacilli and bifidobacteria associated with food have been considered to be safe (Adams and Marteau, 1995). Nevertheless, over the years different side-effects have been related with the use of probiotics, such as systemic infections, deleterious metabolic activities, excessive immune stimulation in susceptible individuals and promotion of deleterious gene transfer (FAO/WHO, 2002). Thus, in the report of FAO/WHO (2002), it was recommended that even when the bacteria have the GRAS or QPS status, before their usage as new probiotics, they should be subjected at least to the following tests:

1. Determination of antibiotic resistance patterns.
2. Assessment of certain metabolic activities.
3. Assessment of side-effects during human studies.
4. Epidemiological surveillance of adverse incidents in consumers (post-market).
5. Assessment of haemolytic activity or toxin production if the strain under evaluation belongs to a species known to possess these activities.

1.1.2. Types of probiotic

Most probiotics are bacteria, with the exception of some yeasts and fungi (FAO, 2016). Among the bacteria, the most representative group of probiotics consists of the lactic acid bacteria (LAB), which include members of the *Lactobacillales* order, and whose most important representatives belong to the *Lactobacillus* genus. Description of this group is presented in detail in section 2 of this introduction. Another very important group of probiotics are members of the *Bifidobacterium* genus, which is naturally abundant in the gastrointestinal tract (GIT), mainly in the colon, of humans and some other animals. Thus, they have been widely studied as probiotics in humans (Sarkar and Mandal, 2016; Linares et al., 2017), carrying out studies with different species of *Bifidobacterium* such as *B. animalis*, *B. bifidium*, *B. bifidus*, *B. thermophilus*, *B. longum*, *B. pseudolongum* and *B. lactis* for application in both humans and animals.

Nowadays, spore forming bacteria, particularly from the *Bacillus* genus, are increasingly being used as probiotics, because of their resistance to physical and environmental factors, such as heat, desiccation and UV radiation (Setlow, 2014), enabling them to maintain their viability during feed pelleting, storage and handling (FAO, 2016). *Bacillus* spores may survive extreme acidity and tolerate bile salts and other hostile conditions of the GIT (Setlow, 2014; Elshaghabee et al., 2017). The most commercialized species are *B. subtilis*, *B. coagulans* and *B. licheniformis*. Some strains have antimicrobial activities producing bacteriocins belonging to the lantibiotics group (Abriouel et al., 2011), such a subtilin, subtilosin or mersacidin produced by *B. subtilis*, or coagulin produced by *B. coagulans*. Also, *Bacillus* strains can produce several vitamins, enzymes and peptides that promote the growth and diversity of beneficial microorganisms in the intestine (Elshaghabee et al., 2017). In addition, there are other spore-forming bacteria belonging to the *Clostridium* genus that are used as probiotics. Among them, *Clostridium butyricum* is the most representative species and is able to produce short-chain fatty acids (SCFA), promote the growth of lactobacilli and bifidobacteria, and inhibit diarrhoea (Yang et al., 2012). This bacterium also has immunomodulatory effects on colon inflammation by suppressing cytokine secretion (Kanai et al., 2015; Cassir et al., 2016).

Another Gram-positive bacterium, *Faecalibacterium prausnitzii* is considered the most abundant bacterium in the human intestinal microbiota (up to 5% of the total bacterial population) and it is also present in the intestine of all animals so far investigated. This bacterium is related to the healthy microbiota and variation in its abundance has been linked to dysbiosis in several human disorders. In the last few years, the bacterium has been intensely studied because of its influence on the immune system and it has been postulated as a potential new probiotic for use in human immune diseases (Miquel et al., 2013).

Several Gram-negative bacteria are considered human and animal pathogens, like enterohemorrhagic *Escherichia coli* strains. But there are non-pathogenic *E. coli* strains living in the intestine as commensal microorganisms. Thus, in 1917 the scientist Alfred Nissle isolated one strain from the faeces of a First World War soldier who did not develop enterocolitis during a severe outbreak of shigellosis (Guarner et al., 2017). This strain named *E. coli* Nissle 1917 was tested against *Salmonella* and other coliform enteropathogens and showed great antagonist activity inhibiting the growth of the pathogens (Sonnenborn, 2016). This strain became one of the most utilized probiotics under the name of *Mutaflor*.

Among the eukaryotic microorganisms used as probiotics in human beings or animals, yeasts are the most representative group. Effects have been reported at the immunological level, against diarrhoea and enteric pathogens, or in maintaining the integrity of the epithelial barrier (Moslehi-Jenabian et al., 2010). For use in human health, *Saccharomyces cerevisiae* and *S. boulardii* are the most commercialized yeasts (Nayak, 2011), and for use in animal feed strains belonging to the genera *Pichia*, *Candida*, *Metschnikowia*, *Yarrowia* or *Debaryomyces* are also used (Vohra et al., 2016). Their probiotic interest is due to their antibacterial properties against pathogens such as *Staphilococcus aureus*, *E. coli*, *Salmonella* sp. or *Pseudomonas aeruginosa* (Seddik et al., 2016) and also for their anti-inflammatory effects, and the production of substances of high biological value such as vitamins, oligoelements or extracellular enzymes such as amylases, β-galactosidases and phytases that promote the digestibility of food (Vohra et al., 2016). Compared to probiotics of prokaryotic origin they have advantages such as their use during antibiotic treatments or the impossibility of transferring antibiotic resistance genes. Nonetheless, they also have disadvantages such as a low ability to produce antagonistic compounds and to colonize the intestine (Nayak, 2011).

1.1.3. Mode of probiotic action

Different probiotics exert their effects through various mechanisms, many not yet fully understood, and even closely related strains can act via different modes of action. Probiotics exert their beneficial effect mainly in the GIT and they are postulated as the main substitute for the use of antibiotics (FAO, 2016). The main mechanisms of action of probiotics (Fig. 1) are to strengthen the intestinal barrier, stimulate the immune system or counteract the effects of pathogens through acidification of the environment, the production of bacteriocins and competition for nutrients and adhesion to the epithelium (Guarner et al., 2017).

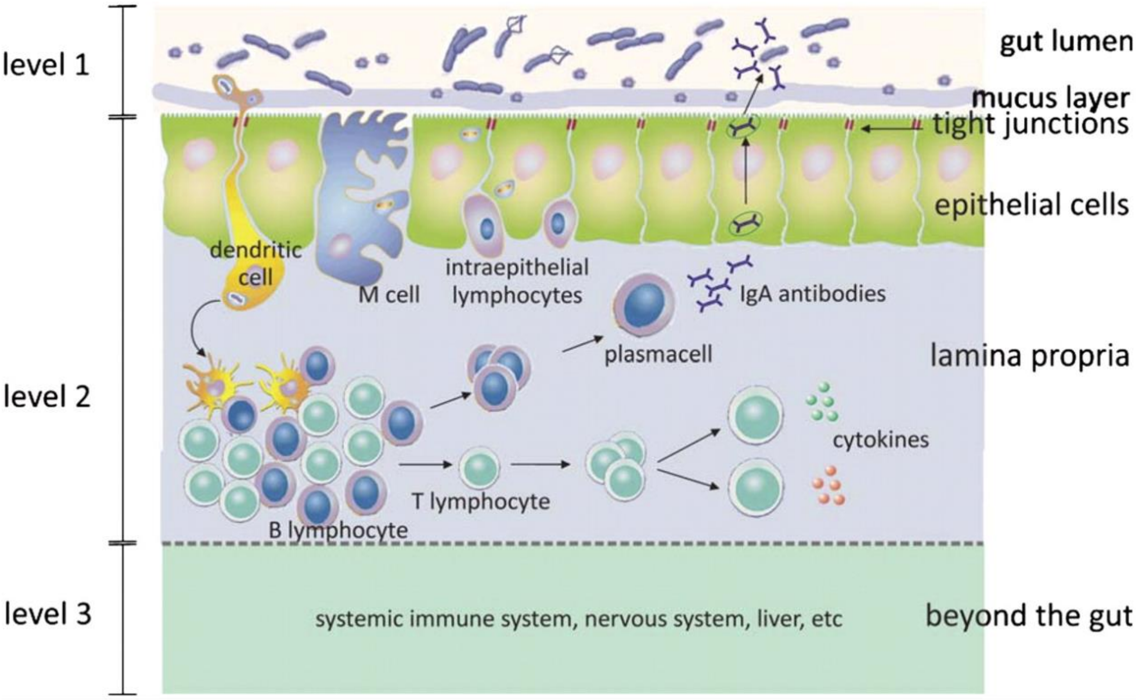

Figure 1. Mechanism of action of probiotic bacteria. They can interfere with growth or survival of pathogenic microorganisms in the gut lumen (level 1). They can improve the mucosal barrier function and mucosal immune system (level 2) and, beyond the gut, they have an effect on the systemic immune system as well as other cell and organ systems such as liver and brain (level 3). Reproduced from Rijkers et al. (2010).

1.1.3.1. Mechanisms against pathogens

Probiotics prevent the process of pathogenicity by inhibiting growth or eliminating pathogenic bacteria from the intestinal lumen through various direct or indirect mechanisms. Probiotics can produce a wide range of molecules that can reduce the number of pathogens by modifying their metabolic or toxin production capabilities (Liao and Nyachoti, 2017). Bacteriocins are antimicrobial peptides synthesized ribosomally by Gram-negative and Gram-positive bacteria (Al Kassaa et al., 2014). They generally have a narrow spectrum of activity and, unlike antibiotics, act on the target pathogenic bacteria without harming the surrounding population. They have non-toxic and non-immunogenic characteristics (Cotter et al., 2012). Some examples of bacteriocins are: (i) plantaricins produced by *Lactobacillus plantarum*, active against *Clostridium*, *Salmonella*, *Staphylococcus* or *Pseudomonas* (Seddik et al., 2017); (ii) pediocins produced by *Pediococcus acidilactici*, active against a large number of Gram-positive pathogen bacteria (Rodríguez et al., 2002); (iii) enterocins produced by *Enteroccus faecium*, active against *Clostridium perfringens*, *S. aureus*, *E. coli* and *Salmonella* (Grande Burgos et al., 2014; Al Atya et al., 2016; Caly et al., 2017) and (iv) salivaricins such as that produced by *Lactobacillus salivarius* B-30514 which inhibits *Campylobacter jejuni* in poultry (Stern et al., 2008).

Organic acids produced by LAB, such as lactic acid or acetic acid, have a broad spectrum of action because they act by reducing intracellular pH and inhibiting active transport, as well as different metabolic functions, and are capable of altering the growth of pathogens (Zhitnitsky et al., 2017). Other type of metabolites such as reuterin, a secondary metabolite derived from the fermentation of glycerol produced by *Lactobacillus reuteri* with a broad spectrum of activity, inhibits DNA synthesis and is resistant to proteolytic and lipolytic enzymes (Singh, 2018).

Furthermore, probiotics may exclude or reduce the growth of other microorganisms in the GIT, either through direct or indirect competition for nutrients or adhesion sites (Oelschlaeger, 2010). LAB probiotics such as *L. plantarum* or *Lactobacillus rhamnosus* GG strains stimulate mucin production in intestinal cells, which prevents the adhesion of pathogenic strains such as *Salmonella*, *Clostridium* and *E. coli* (Oelschlaeger, 2010). By competitive exclusion, lactobacilli are able to displace *Clostridium difficile* or *Vibrio parahemolyticus* (Arruda et al., 2016; Sha et al., 2016). In addition, different lactobacilli strains showed inhibitory activity against a *Salmonella enteriditis* infection and the reduction of its associated damage (Penha Filho et al., 2015). The administration of a *L. salivarius* 59 and *E. faecium* PXN-33 mix obtained a similar effect on a *S. enteriditis* infection (Carter et al., 2017).

1.1.3.2. Stimulation of the immune system

The immunomodulatory effects of probiotics have been proposed as strain specific, and probably also specific for host species (Vlasova et al., 2016). There are many compounds such as carbohydrates, lipids and proteins that are components of microbial structure, mainly the wall or cell membrane, that are known as pathogen-associated molecular patterns (PAMPs). These PAMPs mediate the recognition of the probiotics by the innate immune system through the pattern recognition receptors (PRRs) (Modlin et al., 1999). PRRs are expressed on immune cell and the intestinal epithelium surfaces, and are divided into three main families: Toll-like receptors (TLRs), retinoic acid inducible gene I (RIG-I)-like receptors (RLRs) and nucleotide oligomerization domain-like (NOD) receptors (NLRs). PRRs lead signalling cascades that implement the recruitment of adaptor molecules such as myeloid differentiation primary response gene 88 (MyD88), which in turn activates the mitogen-activated protein kinase (MAPK) pathway and the nuclear factor kappa B (NF-κB) pathway (Wells, 2011). Probiotics can modulate the immune response, through these pathways, stimulating the expression of genes encoding several cytokines such as IL-1β, IL-6, IL-8, IL-12 or IFN-γ by macrophages or dendritic cells (DCs). As a consequence, increased levels of these cytokines are secreted and they trigger the recruitment of other immune cell types, activation of phagocytosis and induction of different T cell subsets (Shida and Nanno, 2008; Wells, 2011; Brisbin et al., 2015; Quinteiro-Filho et al., 2015). In addition, probiotics can also have an anti-inflammatory effect, inhibiting the secretion of pro-inflammatory cytokines and promoting the secretion of the anti-inflammatory IL-10 that induces the maturation of DCs and regulatory T cells (Dwivedi et al., 2016). Among probiotics, LAB have been reported to have a positive effect on the immune system, affecting both innate and adaptive immune responses, modulating the cytokine profiles as describe above, mainly triggered by the TLR2 signalling pathway, and also by the TLR2 receptor in combination with the TLR6 forming heterodimers (Wells, 2011; Tsai et al., 2012; Ashraf and Shah, 2014).

1.1.3.3. Intestinal barrier

The ability of the intestinal epithelium to act as a barrier between the external and the internal environments is absolutely essential for human and animal health, acting as the first defence against pathogens (Arrieta et al., 2006). Thus, when the intestinal barrier is damaged it causes internalisation of the microbial antigenic material, and in addition this event can significantly alter the immunogenicity of the microbial antigens (Madsen, 2012). Intestinal epithelial cells are the first and most important target cells for probiotic action (Lebeer et al., 2008). Probiotic strains have been shown to have beneficial effects related to certain aspects of these epithelial cells. Thus, some metabolites produced by probiotic bacteria, such as butyrate, act as substrates for further metabolism by colon mucosal cells and it has been suggested to be also important for the maintenance of the intestinal epithelium (Donohoe et al., 2011).

Epithelial cells bind to each other through a series of intercellular joints along their lateral margins called tight junctions. These structures are very complex and are composed of lipoid and proteinaceous components. Occludin, claudins, junctional adhesion molecule and tricellulin are the major transmembrane proteins involved in tight junction assembly (Rao and Samak, 2013). Toxins from pathogens target these proteins. There are several studies supporting that some probiotics such as *L. rhamnosus* and *B. lactis* strains can contribute to the epithelial resistance by promoting an increase in the phosphorylation activity over these proteins (Mathias et al., 2010), or other probiotics such as *L. plantarum* strains that promote an increase in the expression levels of certain genes associated with the formation and stabilization of the tight junctions (Anderson et al., 2010).

Probiotics may also improve the function of the intestinal barrier by increasing the production of cytoprotective molecules such as heat shock proteins. These proteins participate in many regulatory pathways and act as molecular adjuvants of other cellular proteins contributing to the maintenance of homeostasis. Certain probiotics such as *L. rhamnosus* GG and *B. subtilis* strains induce the expression of these heat shock proteins (Madsen, 2012). Finally, probiotics can also prevent epithelial damage induced by cytokines and oxidants by promoting cell survival and preventing entry into apoptosis (Madsen, 2012).

1.1.4. Probiotics in the diseases

The probiotics through the mechanisms described above (and others that are still uncertain) are able to mediate in the pathology of various diseases or alterations of human and animal health. Their effects on the treatment of infectious processes such as diarrhoea, by affecting the bacterial homeostasis and the strength of the intestinal barrier are well known (Pattani et al., 2013; Guarino et al., 2015; Goldenberg et al., 2017; Santos do Carmo et al., 2018). The involvement of the probiotics in the modulation of the immune system make them important tools for the treatment of symptoms of various inflammatory diseases: (i) inflammatory bowel diseases (Saez-Lara et al., 2015; Wasilewski et al., 2015; Abraham and Quigley, 2017; Nishida et al., 2018), (ii) celiac disease (Tavakkoli and Green, 2013; de Sousa Moraes et al., 2014), (iii) obesity (Tsai et al., 2014; Borgeraas et al., 2018), (iv) diabetes (Samah et al., 2016), and (v) allergies (Satya et al., 2014; Żukiewicz-Sobczak et al., 2014). Many of these diseases are autoimmune, where chronic inflammation occurs. Prolonged inflammatory events can trigger other diseases such as cancer, and it has been reported that probiotics can exert a beneficial effect to prevent them and to act as adjuvant in their treatment, particularly for colorectal cancer (Ambalam et al., 2016; Yu and Li, 2016; So et al., 2017). In addition, probiotics can affect blood pressure and the serum cholesterol level, which in combination with their benefits in obesity and diabetes are valuable for the treatment of cardiovascular diseases (Ebel et al., 2014; Thushara et al., 2016). In the last years, numerous studies have been performed with the aim to unravel the relationship between intestinal microbiota and nervous functions, the so called “gut-brain axis”. Also, to determine the scientific bases of how the use of probiotics can affect central nervous system disorders such as anxiety depression, obsessive-compulsive disorder, bipolar disorder, autism spectrum disorder and memory abilities (Mangiola et al., 2016; Umbrello and Esposito, 2016; Vlainić et al., 2016; Wang et al., 2016; Rios Adiel et al., 2017).

1.2. Prebiotics

Since the beginning of the 20th century, carbohydrate intake has been associated with the enrichment of *Lactobacillus* and *Bifidobacterium* in human faeces. As already mentioned, many species of these two genera are considered probiotic microorganisms, as since their origins both concepts, probiotic and prebiotic, have been reported. Thus, in 1995 prebiotics were defined as “non-digestible food ingredients that beneficially affects the host by selectively stimulating the growth and/or activity of one or a limited number of bacteria already resident in the colon” (Gibson and Roberfroid, 1995). Over the years, this definition has changed because knowledge of these substances has progressed, always relating their ability to modulate the microbiota with beneficial effects for the host. However, definitions suggested by FAO or Bindels et al. (2015) where limiting the concept to modulate just the gut microbiota and the requirement for selective fermentation was no longer used, provoking disagreements among experts. For this reason, the International Scientific Association for Probiotics and Prebiotics (ISAPP) has made a consensus to define prebiotic as “a substrate that is selectively utilised by host microorganisms conferring a health benefit” (Gibson et al., 2017). First, this definition emphasizes that a prebiotic needs a specific fermentation by a limited range of microorganisms that could affect composition of the microbiota. Thus, other substances, such as antibiotics, vitamins or minerals that can affect microbiota in a generalized way, are excluded by definition. On the other hand, this definition includes action areas of prebiotics beyond the GIT such as the vaginal tract and the skin (Gibson et al., 2017).

1.2.1. Types of prebiotics and their mode of action

The best recognised prebiotics are the galactooligosaccharides (GOS), the fructo-oligosaccharides (FOS) and inulin-type fructans, which have been validated by numerous studies. Human milk oligosaccharides are very important, particularly in the new-born where they are essential in the election of the microbiota and the correct development of the metabolic and immunological systems (Oozeer et al., 2013; Garrido et al., 2015). Bifidobacteria preferentially ferment these types of oligosaccharides and others such as xylooligosaccharides (XOS), which is why these carbohydrates promote the so-called bifidogenic effect of probiotics. In addition, the fermentation of these oligosaccharides promotes the formation of SCFA with acetic, propionic and butyric acids being the most abundant. These SCFA can affect locally the gut health, but also can serve as signalling molecules, or enter the systemic circulation and exert their benefits in other places (Canfora et al., 2015; Rastall and Gibson, 2015; Sun et al., 2017). Other carbohydrates such as lactulose or certain fermentable fibres are considered prebiotics, as well as different types of polysaccharides produced by bacteria. Furthermore, there are reports showing that non-carbohydrate substances such as polyphenols and polyunsaturated fatty acids exert prebiotic effects (Peluso et al., 2014; Duda-Chodak et al., 2015; Moreno-Indias et al., 2016; Costantini et al., 2017).

Prebiotics can exert their effects through mechanisms of action similar to those of probiotics and affect similar processes of health alteration. For example, scientific reports, mainly performed with FOS and GOS, have revealed the health effect of prebiotics on primarily GIT: (i) by helping to maintain homeostasis by microbiota modulation, (ii) by preventing or acting on dysbiosis, (iii) by inhibiting pathogens colonisation and (iv) by contributing to the treatment of the symptoms of inflammatory bowel diseases as well as to the prevention of colorectal cancer (Ambalam et al., 2016; Louis et al., 2016; Holscher, 2017; Lane et al., 2017; Rasmussen and Hamaker, 2017). In addition, prebiotics have also positive effects on human metabolism contributing to reduce blood lipid levels as well as to modulate the factors involved in obesity, insulin resistance and the metabolic syndrome (Beserra et al., 2015; O’Connor et al., 2017; Choque Delgado and Tamashiro, 2018). Moreover, they have a positive effect on: (i) the bones, by increasing the bioavailability of minerals (McCabe et al., 2015; Whisner and Castillo, 2018), (ii) the nervous system modulating behaviour and reducing anxiety and stress (Kao et al., 2016; Burokas et al., 2017), and (iii) the prevention and relief of allergies and skin diseases such as eczema and acne (Osborn and Sinn, 2013; Baquerizo Nole et al., 2014; Boyle et al., 2016; Cuello-Garcia et al., 2017).

1.2.2. Prebiotics as immunomodulators

Section 1.1.3.2 has already described how one of the most important beneficial actions of probiotics is to modulate the immune system, making these microorganisms, mainly bacteria, very important immunomodulators. These immunomodulatory responses are due to the recognition of PAMPs by the host (Wells, 2011). Prebiotics, are mainly carbohydrate compounds that can be recognized by the PPRs and exert some of their reported benefits through immune system modulation.

1.2.2.1. Oligosaccharides

FOS, GOS and XOS, as well as other olygosaccharides, promote anti-inflammatory effects by acting on the expression of cytokines by the immune cells. This effect is to provoke either reduction of secretion of pro-inflammatory cytokines (e.g. IL-1β, IL-6, IL-8, IL-12, TNFα and IFN-γ), or increase of release of anti-inflammatory cytokines (e. g. IL-10 and IL-13) (Hoentjen et al., 2006; Vulevic et al., 2008; Lecerf et al., 2012; Dehghan et al., 2014). In addition, the fermentation of prebiotics by the microbiota can result in the production of compounds such as SCFA that in turn can affect the immune system. SCFA are absorbed in the gut and can modulate immune cells of the gut-associated lymphoid tissues (GALT), by binding to the G-protein-coupled receptors GPR41 and GPR43 (Brown et al., 2003a). SCFA, mainly butyrate, reduce TNFα production and inhibit expression of IL-1β and IL-6 genes via the NF-κB pathway (Segain et al., 2000; Tedelind et al., 2007). Another study reported that butyrate reduces pro-inflammatory IL-2 and IFN-γ production, while acetate and propionate increase anti-inflammatory IL-10 production (Cavaglieri et al., 2003).

1.2.2.2. β-glucans from eukaryotic organisms

β-glucans from yeast, fungal and plant cell wall have been reported as interesting prebiotics for the development of functional foods (Lam and Chi-Keung Cheung, 2013; Arena et al., 2016). They are non-digestible carbohydrates of high molecular weight, which have immunomodulatory properties. In ancient oriental cultures such as those developed in China or Japan, they used extracts of mushrooms and plants to treat a wide variety of diseases, and a large part of that potential is due to the glucans contained in those extracts (Novak and Vetvicka, 2008; Martel et al., 2017). β-glucans seem to activate the immune response against various infections, exert numerous beneficial effects on: (i) cardiovascular diseases, (ii) metabolic diseases such as diabetes and obesity, (iii) inflammatory processes and their related diseases as well as acting as anticancer agents (Chen and Seviour, 2007; Chan et al., 2009; Driscoll et al., 2009; Du et al., 2015; Zhu et al., 2015; Baldassano et al., 2017; Jayachandran et al., 2018; Jin et al., 2018).

Depending on the β-glucan source, they can be composed of β-(1,3) and/or β-(1,6)- and/or β-(1,4)-linked β-D-glucopyranose units, and they can present side chains with β-(1,3) or β-(1,6)-linkeages (Wasser, 2002; Synytsya and Novák, 2013). For example, cereal (oat and barley) β-glucans are linear β-(1, 3) and β-(1, 4)-linked (Daou and Zhang, 2012; Shah et al., 2017), while fungal β-glucans are usually linear β-(1, 3) with or without branching β-(1, 6)-linkeages (Chen and Seviour, 2007; Zhu et al., 2015). Because of their complexity in composition, length and structure, β-glucans can be recognized by different kind of PRRs (Fig. 2) located on the surface of several immune cells such as monocytes, macrophages, DCs, neutrophils and natural killers (Chan et al., 2009; Turvey and Broide, 2010). Thus, the main receptors involved in the recognition of β-glucans are dectin-1, toll-like receptors (TLRs), complement receptor 3 (CR3), lactosylceramide (LacCer) and scavenger receptors (SRs).

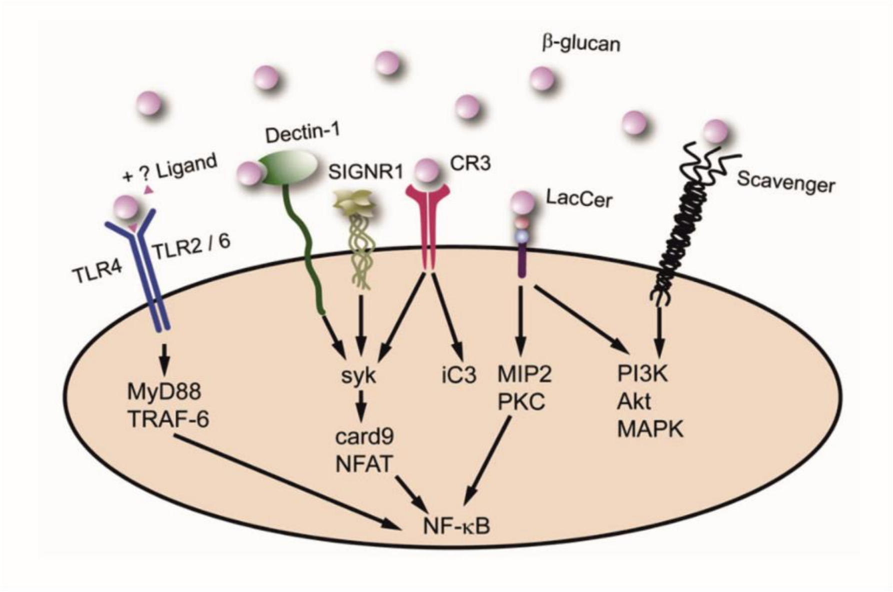

Figure 2. Receptors for the recognition of β-glucans. Reproduced from Chan et al (2009).

Dectin-1 is a small type II transmembrane receptor containing a single extracellular C-type lectin-like domain (CTLD), which recognizes β-(1, 3) and/or β-(1, 6)-linked glucans and intact yeasts. CTLD is involved in calcium-independent ligand interactions, connected to a single-pass transmembrane domain by a stalk region (Brown and

Gordon, 2001; Taylor et al., 2002; Plato et al., 2013). Although its name originates from "dendritic-cell-associated C-type lectin 1", dectin-1 is not exclusively expressed on DCs, indeed it can be found expressed at higher levels on other immune cell types including macrophages, neutrophils and monocytes (Taylor et al., 2002; Brown et al., 2003b). Dectin-1 can recognize fungi belonging to several genera, through their β-glucans, such as *Aspergillus*, *Candida*, *Coccidioides*, *Penicillium*, *Pneumocystis* and *Saccharomyces*. Focus on its immunological role, the signalling route through dectin-1 can induce a vast array of cellular effects including actin-mediated phagocytosis, activation of the respiratory burst through production of reactive oxygen species (ROS), endocytosis, DCs maturation and changes in cytokine and chemokine expression patterns (Brown, 2005; Huysamen and Brown, 2009; Plato et al., 2013). To carry out these innate responses requires TLRs to generate pro-inflammatory responses (Brown et al., 2003b). In addition, dectin-1 is able to activate the CR3, resulting in a combined signalling to enhance phagocytosis and ROS production by neutrophils at the site of infection (Li et al., 2011; Plato et al., 2013).

The TLRs seem to be the primary sensing receptors of the innate immune system and are involved in the defence against invading bacteria, fungi and viruses, among other pathogens. TLRs are the most extensively studied family of PRRs and, to date, 13 members have been identified. TLRs are type I transmembrane receptors containing a horseshoe-shaped ligand-binding domain (LBD) and a signalling tail connected by a transmembrane region. TLR signalling often results in activation of NF-κB and MAPKs, both major components of the regulation of gene expression (Leulier and Lemaitre, 2008; Yamamoto and Takeda, 2010; Plato et al., 2013).

CR3 is a specific receptor of the complement system that takes part in cellular immunity. CR3 is a heterodimer integrin composed of α and β chains, activated by the carbohydrate present on the surface of a bacterium, a virus or a transformed cell. Its activation induces inflammatory responses including chemotaxis of different lymphatic cells such as neutrophils, eosinophils, basophils and macrophages. The function of CR3 has been extended to include adhesion, activation of oxidative burst, cytokine production and cytotoxicity (Yefenof, 2002).

LacCer is a glycosphingolipid found in the plasma membranes of many cells and was identified as a β-glucan receptor from biochemical analyses of the interactions between β-glucan and isolated human leukocyte membrane components (Zimmerman et al., 1998).

SRs are present on the surface of a variety of cell types and although typically associated with cholesterol metabolism, also recognize and respond to pathogens. SRs present in macrophages and monocytes recognize a variety of PAMPs including β-glucans. SR interactions with carbohydrate polymers depend on the structure, but also on charges and other structural determinants (Gough and Gordon, 2000; Rice et al., 2002).

1.3. Evaluation of probiotics and prebiotics

Probiotics and prebiotics are formulated to be used in both human beings and animals, as part of functional foods or administered as drugs. For this reason, before they can be commercialised, a validation of their safety and their action on health has to be performed. Moreover, the evaluation of new probiotics and prebiotics should follow an order of complexity, starting with simple *in vitro* models, then moving to *in vivo* models and ending with human trials.

1.3.1. In vitro assessment

The first step in the evaluation of a probiotic is to assess its safety, which involves assessing its antibiotic resistance profile and its ability to produce substances (e. g. (biogenic amines), enzymes or toxins (e. g. proteases, haemolysins or cytolysins) that could negatively affect its potential host. These characteristics can be assessed by the use of growth techniques in the presence of antibiotics and detection of compounds by chromatographic techniques. For antibiotic resistance, EFSA has determined the minimum inhibitory concentration (MIC) for the most commonly used antibiotics (EFSA, 2012).

In addition, *in vitro* techniques are used for the detection of compounds with antimicrobial activity, such as the production of organic acids or antimicrobial peptides. These techniques consist of inhibition tests using the strain or its supernatant against the selected pathogens. Tests with neutralized supernatants are carried out for the detection of antimicrobial compounds such as bacteriocins, whose effect is not due to a decrease of the pH produced by the bacterial metabolism. Subsequently, the characterisation of these compounds is carried out using chromatographic techniques.

1.3.1.1. In vitro GIT simulation models

As already mentioned, probiotics must reach, alive, the site of action, that is usually the colon, to exert their beneficial effects. To do this, they must resist the stresses associated with the GIT, both the action of pH and the action of different enzymes. Prebiotics must also survive the action of these agents, to arrive intact and be fermented by the intestinal microbiota. To test bacterial resistance, there are models where the simulated stresses are evaluated independently or sequentially by direct addition of the stressors: (i) the oral stress subjecting the bacteria to lysozyme, (ii) gastric stress subjecting the bacteria to low pH and the action of pepsins, and (iii) intestinal stress subjecting the bacteria to bile salts and pancreatic juices (Fernández de Palencia et al., 2009). More sophisticated models have also been developed, in which the human GIT is simulated in an autonomous way, each section (oral cavity, stomach, and diverse regions of the intestine) is simulated in compartments connected to each other. The probiotics or prebiotics pass sequentially, and all the parameters are computer-controlled. In addition, these systems can harbour a complex microbiota, that in some cases try to represent the common composition of particular regions of the colon, or even the whole human gut, to evaluate the effect of the probiotics and prebiotics on the microbial environment (Marteau et al., 1997; Lo Curto et al., 2011; Sarbini et al., 2011). The most used is the simulator of the human intestinal microbial ecosystem (SHIME), that consists in a five-stage reactor that simulates the small intestine by a two-step (duodenum-jejunum and ileum) system and the large intestine by a three-step (ascending, transverse and descending colon) reactor (Molly et al., 1993). This model has been widely used and updated including a stomach-step (Alander et al., 1999) or recently incorporating a mucosal environment, containing mucin-covered microcosms, to assess lactobacilli colonization (Van den Abbeele et al., 2012).

1.3.1.2. In vitro adhesion and immunomodulation models

Probiotics need to extend their residence time in the GIT in order to modulate the microbiota. To this end, they must be able to interact with the intestinal mucosa and epithelium. In addition, this type of interaction, of both probiotics and prebiotics, is necessary to exert their modulation of the immune system. In order to evaluate the adhesion capacity of probiotics two models are currently used either: (i) models containing mucin absorbed onto abiotic surfaces like polystyrene (Jonsson et al., 2006; Ruas-Madiedo et al., 2006; Laparra and Sanz, 2009; Arboleya et al., 2011) or (ii) confluent intestinal cell layer cultures, mainly the Caco-2 and the HT-29 human cell lines derived from carcinoma, and also the mucus overproducing HT-29-MTX cell line (Fernández de Palencia et al., 2009; Arboleya et al., 2011; de los Reyes-Gavilán et al., 2011; Archer et al., 2018). However, there is no standard procedure for testing bacterial adhesion, since the first type emphasizes more the interaction with mucus and the other emphasizes more direct host-microbe cellular interaction, and wide variation between models can be found. For this reason, more complex models could be used like the co-culture of Caco-2 and HT29-MTX cell lines that reproduces a more physiological cell-type ratio of mucosal and epithelial tissues (Chen et al., 2010; Béduneau et al., 2014); or even, fragments of colonic tissue excised from the host can be used for *in vitro* adhesion studies where, in addition to the mucosal layer and epithelial tissue, the interaction with the commensal microbiota is taken into account (Henriksson et al., 1991; Vesterlund et al., 2005). In addition, an *in vitro* cellular line model can be used to test competition activity of probiotics against intestinal pathogens such as *Listeria monocytogenes* (Garriga et al., 2014; Winkelströter and De Martinis, 2015), *E. coli* (Gopal et al., 2001; Candela et al., 2008) or *Salmonella typhimurium* (Candela et al., 2008); and also to study their effects on cytotoxicity produce by the toxins of *C. difficile* (Valdés-Varela et al., 2016).

In order to study the immunomodulatory characteristics of probiotics and prebiotics, it is possible to analyse the response they provoke on epithelial cell lines such as HT-29 (López et al., 2012; Patten et al., 2014; Sánchez et al., 2015) or Caco-2 (Yanagihara et al., 2012; Wang et al., 2017). Also, there are several human monocyte cell lines that can be used for this purpose. The most common is the THP-1 cell line due to its similarity with human macrophages derived from monocytes. THP-1 differentiation to macrophages is induced by exposure to phorbol 12-myristate 13-acetate (PMA), and this model has been used to assess the effect on cytokine profile exerted by bacteria (Yanagihara et al., 2014) and carbohydrate compounds (Notararigo et al., 2014; Gill et al., 2016; Balzaretti et al., 2017). In addition, human monocytes isolated from buffy coats can be differentiated to pro-inflammatory M1 macrophages or anti-inflammatory M2 macrophages, by exposure to granulocyte-macrophage colony-stimulation factor (GM-CSF) or macrophage colony-stimulation factor (M-CSF) respectively, and used as an *in vitro* immunomodulation model (Fernández de Palencia et al., 2009; Garai-Ibabe et al., 2010; Notararigo et al., 2014). Moreover, mouse peritoneal macrophages are commonly used as an *in vitro* model (Ciszek-Lenda et al., 2011; Kang et al., 2011). Finally, the control of intestinal homeostasis is closely linked to the communication between epithelial cells and immune cells from the lamina propia, acting on the integrity of the intestinal barrier and on the transduction of the immune response. Transwell *in vitro* models try to mimic this environment by using co-cultures of epithelial cells such as Caco-2 in the upper compartment and cultures of macrophages or DCs in the lower compartment. This model is ideal to study the signal transduction triggered by probiotics and prebiotics (Bermudez-Brito et al., 2015; Brufau et al., 2017; Kishimoto et al., 2017).

1.3.2. In vivo animal models

Before performing human trials to evaluate the effects and safety of probiotics and prebiotics, the usage of animal models is required that provide a complex environment and a global response to them. For an animal model to be valid, the mechanisms involved in the response studied must be scientifically correlated with the mechanisms in humans, and also it is essential in the use of *in vivo* models, to adhere to the application of the 3Rs (Replacement, Reduction and Refinement), which supports animal welfare (Sneddon et al., 2017).

Rodents, mainly rat and mice, are the most used animal models. There are many lineages available as well as knock-out models that are of great interest, e. g. an IL-10 knock-out model that produces a chronic intestinal inflammation can be useful to evaluate the effect of probiotics and prebiotics (Ganesh et al., 2012). Inflammatory processes can be induced by exposure to agents such as dextran sodium sulphate (DSS) and 2,4,6-trinitrobenzene sulfonic acid (TNBS), providing effective models to study the anti-inflammatory effects of bacteria and immunomodulatory compounds (Lavi et al., 2009; Dogi et al., 2016; Hidalgo-Cantabrana et al., 2016; Lim et al., 2017; Panpetch et al., 2017). In addition other models that recreate various diseases such as obesity (Dardmeh et al., 2017), diabetes (Li et al., 2016b) and cancer (Li et al., 2016a) may also be appropriate. *Citrobacter rodentium* is a murine enteric pathogen used to generate a diarrheal infective model, in which protective effects of certain strains of *Lactobacillus* have been observed (Rodrigues et al., 2012; Kumar et al., 2016). Other rodent models, such as guinea pig (Tsunemine et al., 2011), rabbit (Copeland et al., 2009), hamster (Huang et al., 2015) or chinchilla (Nhan et al., 2017) can be used. Nevertheless, rodent models have limitations due to several important physiological and metabolic differences with human beings. Thus, pig is the current best *in vivo* model due to the physiological similarity, in terms of digestive and associated metabolic processes, with humans (Heinritz et al., 2013). However, this model is more expensive, the number of individuals is more limited and the ethical considerations more severe. Thus, simpler animal models, such as the *Caenorhabditis elegans* (Clark and Hodgkin, 2013) and *Drosophila melanogaster* (Daisley et al., 2018) invertebrate models, or the zebrafish model are more suitable for initial stages of evaluation.

1.3.2.1. Zebrafish model

Danio rerio is a small subtropical freshwater fish that belongs to the *Cyprinidae* family. This fish is commonly found in shoals of 5-20 individuals in slow-moving or standing waters of rivers and ponds with aquatic vegetation. Its optimal temperature is about 28 ºC, but can tolerate a range of 15-35 ºC, and they usually occur in water with a slightly alkaline pH of 8 and with a salinity range of 0.25-0.75 ppt (Spence et al., 2007; Ribas and Piferrer, 2014).

One of the advantages of the zebrafish as an animal model is its life cycle (Fig. 3), having a very fast embryonic development (Ribas and Piferrer, 2014). The hatching of eggs occurs between 60-72 hours after fertilization and at 120 hours most of their organs are already functional. They are considered embryos until they are able to feed actively, around 96-120 hours, earlier they feed from the yolk sac. They reach sexual maturity at 2-3 months of age. Females can spawn every 2-3 days and a single clutch may contain several hundred eggs. All these characteristics, within the small space required for breeding (adult size is about 4 cm) make this model very advantageous. In addition, according to the EU directive 2010/63/EU, zebrafish embryos are considered to be a suitable alternative animal model, so their use is not restricted by regulation for animal welfare (Strähle et al., 2012).

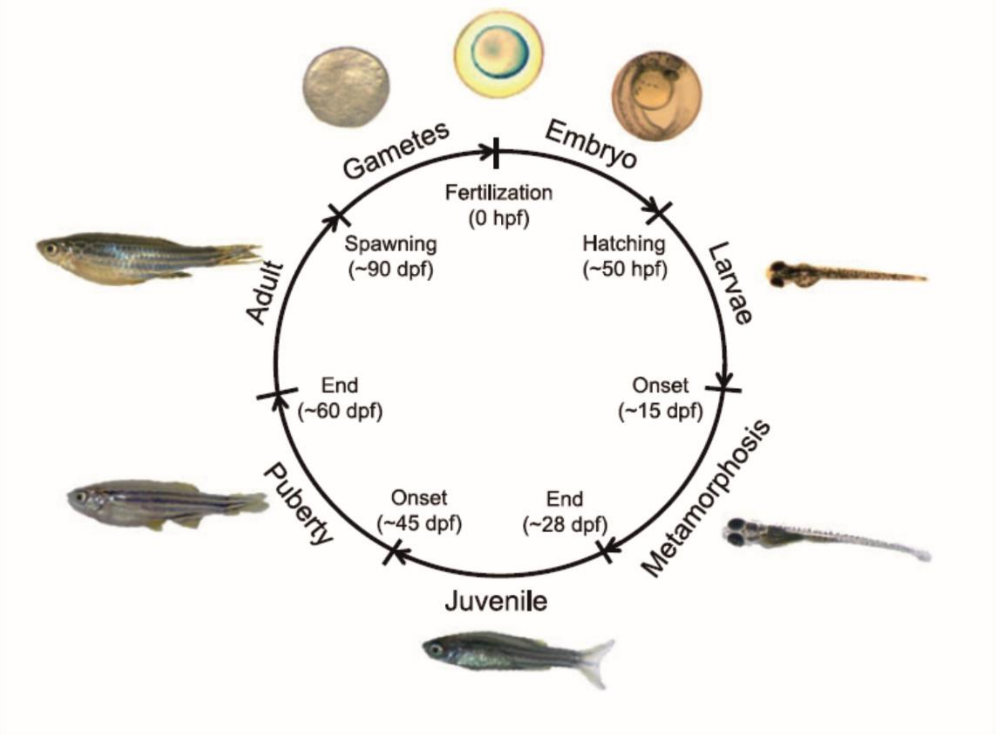

Figure 3. Vital cycle of the zebrafish. All the stages of development from the fecundation to the reproduction age are depicted. Reproduced from Ribas and Piferrer (2014).

Although the zebrafish is a non-mammal model, it possesses important similarities with the human digestive and immune systems (Farber et al., 2001), which makes it a useful model. Except for a functional stomach, the main digestive organs are present in the zebrafish such as the intestine, liver, pancreas and gallbladder. The intestine is divided into three regions (intestinal bulb, mid intestine and posterior intestine) which resemble the small and large intestines of mammals (Wang et al., 2010). Enterocytes, endocrine cells and mucus-producing goblet cells are present in the intestinal epithelium. Adjacent to this epithelial layer is the lamina propia, which harbours several cellular types involved in gut immunity such as macrophages and neutrophils. The zebrafish has innate and adaptive immune systems equivalent to mammals (Trede et al., 2004), but the adaptive system does not develop until the 4-6 week of life, this fact allows the study of the innate response during an inflammatory process without the intervention of the adaptive immunity. In zebrafish there also exist PRRs like TLR receptors, adaptor proteins like MyD88, signalling pathways like NF-κB pathway and several cytokines homologous to those from mammalians. Thus, the zebrafish model seems to be very attractive for the evaluation of the influence of probiotics and prebiotics on the health and immune response.

2. Lactic acid bacteria

LAB are a heterogeneous group of Gram-positive bacteria with less than 50 mol % G+C content in their DNA, thus they are included in the *Firmicutes* phylum. LAB are characterized by being coccus- or rod-shape bacteria, non-spore-forming, sessile, catalase negative, oxidase negative, acid-tolerant, aero-tolerant, facultative-anaerobic and with a strictly fermentative metabolism which generates lactic acid as a major metabolite. Normally, LAB are mesophilic bacteria with growth temperature ranging from 20 to 37 ºC, but there are several LAB like *Streptococcus thermophilus* that are considered as thermophilic bacteria with optimum growth temperature ranging from 40 ºC to 45 ºC. LAB have several nutritional requirements for grow such as vitamins, aminoacids or nitrogen bases. Nevertheless, they can metabolise a great number of different carbohydrates and resist stressful conditions (e. g. osmotic, oxidative and acidic shocks) that lets them adapt to a wide range of habitats (van de Guchte et al., 2002; Gänzle, 2015). Because of their versatility, they can be found in many different environments like food (fermented meat and vegetable products, dairy products, sourdough, beverages or ensilages), plants, residual waters, as well as in the gastrointestinal, genital and respiratory tracts of human beings and other animals (Leroy and De Vuyst, 2004; Holzapfel and Wood, 2014).

They are widely known for their industrial applications as food fermentation starters, biocontrol agents, and probiotics. Moreover, they can produce a wide quantity of different metabolites with application in the food and pharmaceutical industries (Leroy and De Vuyst, 2004; Mazzoli et al., 2014). Traditionally, the most important application of LAB is in the dairy industry, but they are also utilised at industrial scale for the fermentation of other food raw materials such as meat and vegetables. Many LAB are considered safe following the criteria discussed in the probiotics section, and are therefore included in the list of microorganisms with QPS status by EFSA (Ricci et al., 2017). Besides, all lactobacilli are generally considered to be QPS. Finally, LAB are considered as important candidates for metabolic engineering strategies due to several well-characterized properties, including knowledge of the genomes of industrial bacteria belonging to *Lactococcus lactis* or *Lactobacillus* species (Gaspar et al., 2013).

2.1. Taxonomy

At the beginning of the 20th century, Orla-Jensen (1919) described how LAB were able to acidify milk, correlating these bacteria with lactic acid production and establishing this fact, along with other characteristics, as rules for their classification. This included such criteria as cellular morphology, mode of glucose fermentation, range of growth temperature, and sugar utilization patterns. Using this classification, the LAB were grouped into four genera: *Lactobacillus*, *Leuconostoc*, *Pediococcus* and *Streptococcus*. Subsequently several researchers revised the taxonomy by inclusion of several more genera (Holzapfel and Wood, 1995; Garrity and Holt, 2001; Ludwig et al., 2009; Holzapfel and Wood, 2014). Recently, Ludwing et al. (2009) based on the analysis of the 16S rRNA genes sequences, postulated the LAB taxonomy including 38 genera clustered into 6 families (Fig. 4) belonging to the phylum *Firmicutes*, class *Bacilli*, and order *Lactobacillales*.

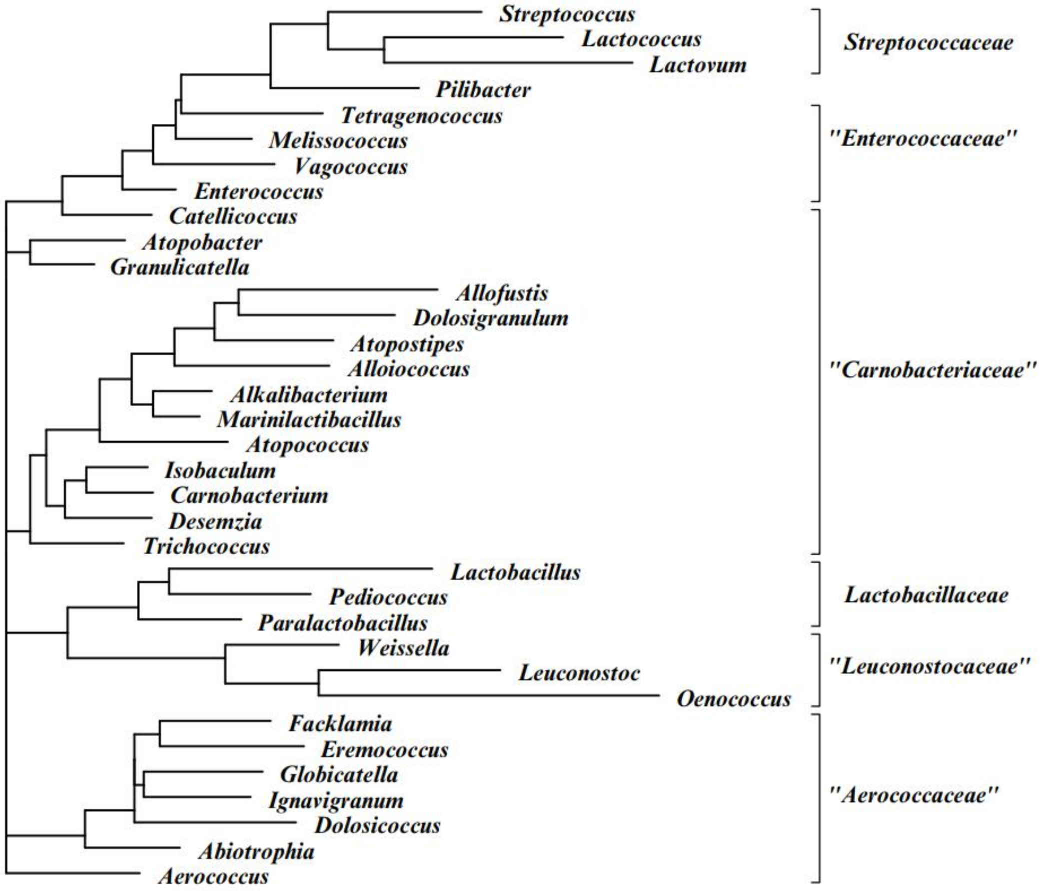

Figure 4. Consensus dendrogram reflecting the phylogenetic relationships of the order *Lactobacillales*. Reproduced from Ludwing et al. (2009).

Currently, on specialised websites such as https://www.ezbiocloud.net/ and http://www.bacterio.net/ new genera such as *Pisciglobus*, *Lactosphaera* or *Bavariicoccus* have been added to the LAB group, reaching a total of 44 genera. Traditionally, the bifidobacteria were included in the *Lactobacillus* genus, based on physiological and metabolomic criteria. Moreover, the bifidobacteria were considered LAB due to sharing with this group certain characteristics such as the production of lactic acid. However, molecular and phylogenetic studies, mainly by rRNA 16S gene sequencing, revealed that bifidobacteria, which have a mol % G+C content higher than 50%, belong to the *Actinobacteria* phylum (Holzapfel and Wood, 2014).

2.2. Carbohydrate metabolism

LAB display a relatively simple carbon and energy metabolism in which the sugar source is converted mainly to lactic acid. From the point of view of their hexose fermentation metabolism, LAB are classified as either homofermentative (producers of only lactic acid) or heterofermentative (producers of lactic acid and other metabolites) (Fig. 5). Previously, it was assumed that LAB obtain energy only by fermentation, because they do not have porphyrins and cytochromes, thus they do not obtain energy from the phosphorylation by the electron transport chain (Pessione, 2012; Von Wright and Axelsson, 2012). However, in the last decades, it was demonstrated that certain bacteria like the homofermentative *L. lactis* and some heterofermentative lactobacilli can take heme and menaquinone from the culture medium and in aerobic conditions are able to obtain energy more efficiently by a respiratory pathway (Pedersen et al., 2012; Gänzle, 2015; Zotta et al., 2018).

Nevertheless, in this review we will only describe in detail the fermentative capacities of LAB. The homofermentative (or homolactic) pathway is based on glycolysis (or Embden–Meyerhof–Parnas pathway), where the fructose-1,6-bisphosphate is obtained by the action of a fructose-1,6-bisphosphate aldolase, and yield a glyceraldehyde-3-phosphate and a dihydroxyacetone-phosphate (Figure 5A). In this pathway, the unique end-product is lactic acid (two molecules for each molecule of hexose) and generate 2 ATP molecules (Von Wright and Axelsson, 2012). In the heterofermentative (or heterolactic) pathway, a 6-phosphate-gluconate is produced by the dehydrogenation of the hexose, and then it is transformed into xylulose-5-phosphate that by the action of a xylulose-5-phosphate phosphoketolase, and yield a glyceraldehyde-3-phosphate and an acetyl-phosphate (Figure 5B). In this pathway, the end-products from each molecule of hexose are one molecule of lactic acid, one molecule of ethanol and one molecule of carbon dioxide. The ATP yield of the heterofermentative pathway is one molecule per hexose molecule, but if acetyl-phosphate is converted to acetic acid instead of ethanol, an extra ATP molecule is formed (Von Wright and Axelsson, 2012).

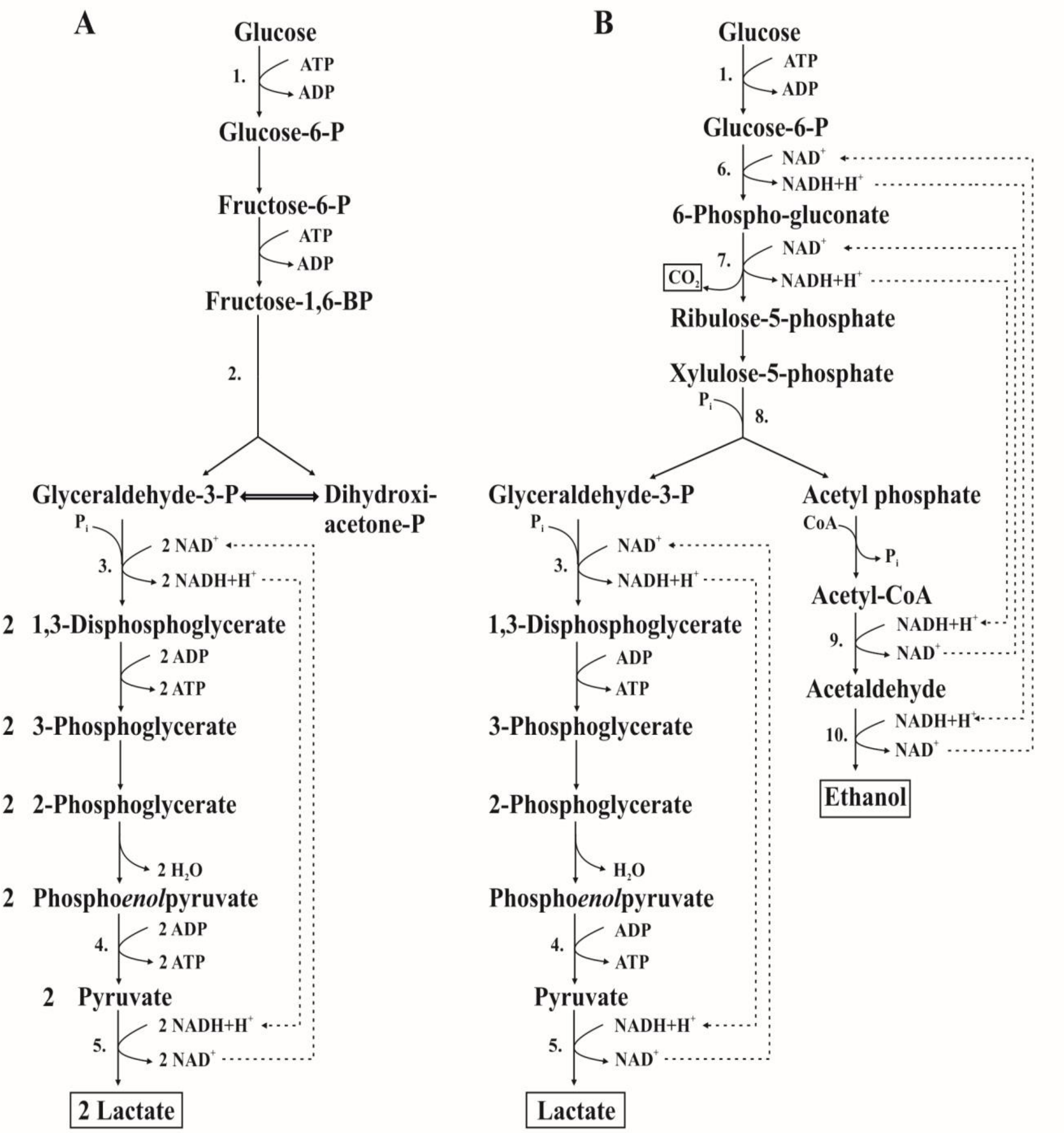

Figure 5. Major fermentation pathways of glucose. (A) Homolactic fermentation and (B) Heterolactic fermentation. Selected enzymes are numbered: 1. Glucokinase; 2. Fructose-1,6-bisphosphate aldolase; 3. Glyceradehyde-3-phosphate dehydrogenase; 4. Pyruvate kinase; 5. Lactate dehydrogenase; 6. Glucose-6-phosphate dehydrogenase; 7. 6-Phospho-gluconate dehydrogenase;8. Phosphoketolase; 9. Acetaldehyde dehydrogenase; 10. Alcohol dehydrogenase. Reproduced from Von Wright and Axelsson (2012).

Generally, pentoses are utilised only in the heterolactic metabolism, by entering as either ribulose-5-phosphate or xylulose-5-phosphate (in this case carbon dioxide is not produced). Nevertheless, there exist examples of homolactic metabolism of pentoses via the pentose phosphate pathway (Tanaka et al., 2002; Gänzle, 2015).

Other hexoses like fructose, galactose and mannose can be isomerized or phosphorylated to either glucose-6-phosphate or fructose-6-phosphate and then enter into one of the above pathways for their metabolism (Pessione, 2012; Von Wright and Axelsson, 2012). Usually, these hexoses like glucose are internalized into the cell by specific membrane transports as permeases or symporters with no ATP waste in membrane translocation, but ATP is needed for the intracellular generation of glucose-6-phosphate or fructose-6-phosphate. In addition, the hexoses can also be transported by a phosphoenolpyruvate (PEP): sugar phosphotransferase system (PTS), that couple carbohydrate translocation and phosphorylation (see details in the next section). Thus, for example depending on how galactose enters in the cell will be differentially metabolised. If it is transported by a permease, galactose will follow the Leloir pathway via galactose-1-phosphate, whereas if it is transported by a PTS system galactose will follow the tagatose-6-phosphate pathway *via* galactose-6-phosphate (Pessione, 2012; Von Wright and Axelsson, 2012).

Thus, depending on the metabolic pathways that they use, LAB are generally clustered in three groups (Von Wright and Axelsson, 2012; Gänzle, 2015):

a) Obligate homofermentative. These bacteria lack the phosphoketolase, thus are unable to metabolize pentoses. They have a constitutive fructose-1,6-bisphosphate aldolase and use only the glycolysis route. LAB belonging to genera *Pediococcus*, *Lactococcus*, *Streptococcus* and Group I lactobacilli (*L. acidophilus, L. delbrueckii*, *L. helveticus* and *L. salivarius*) are included.
b) Obligate heterofermentative. Contrary to the last group these bacteria lack the fructose-1,6-bisphosphate aldolase, having a constitutive phosphoketolase, so they use only the 6-phospho-glucanate route to metabolize hexoses. LAB belonging to the genera *Leuconostoc*, *Oenococcus*, *Weisella* and Group III lactobacilli (*L. brevis*, *L. buchneri*, *L. fermentum* and *L. reuteri*) are included.
c) Facultative heterofermentative. These bacteria metabolize hexoses by homolactic pathway, and pentoses by heterolactic pathway. The 6-phosphogluconate route is inhibited by glucose in these bacteria. Member of the

Group II lactobacilli (*L. casei*, *L. curvatus*, *L. plantarum* and *L. sakei*) and other LAB are included.

In addition, pyruvate has a central role in the fermentation pathways, acting as an electron acceptor to form primarily lactic acid, but depending on the availability of oxygen and substrates, pyruvate can have different fates. Pyruvate could be the substrate of different enzymes like, lactate dehydrogenase, pyruvate dehydrogenase, pyruvate oxidase or pyruvate-formate lyase, therefore many different end-products could be formed such as lactate, acetate, formate, acetyl-CoA, acetoin, ethanol, diacetyl or 2,3-butanediol (Von Wright and Axelsson, 2012; Gänzle, 2015)

2.2.1. PTS system

The PTS is the main system of sugar transport in LAB fermenting hexoses via the Embden–Meyerhof pathway (homolactic and facultative heterolactic LAB). It is characterized by linking the sugar translocation with its phosphorylation using the energy from the hydrolysis of PEP (Postma et al., 1993; Boels et al., 2001).

The PTS system is a multienzymatic system composed of two common cytoplasmic enzymes (EI and HPr) and of different membrane-associated enzymatic complex (EII), specific for each carbohydrate or groups of carbohydrates. The EII component has several domains which can be part of one polypeptide (IIABC), two polypeptides (IIA and IIBC), or several polypeptides (IIA, IIB, IIC and in some cases IID too) and among them at least IIC and IID are bound to the membrane. All of these components form a phosphorylation cascade to transport the phosphate group from PEP to the sugar through transfer to EI, HPr, EIIA and EIIB (Fig 6). EIIC component (sometimes together with EIID) constitutes a one-way transport or permease, which manages the sugar translocation.

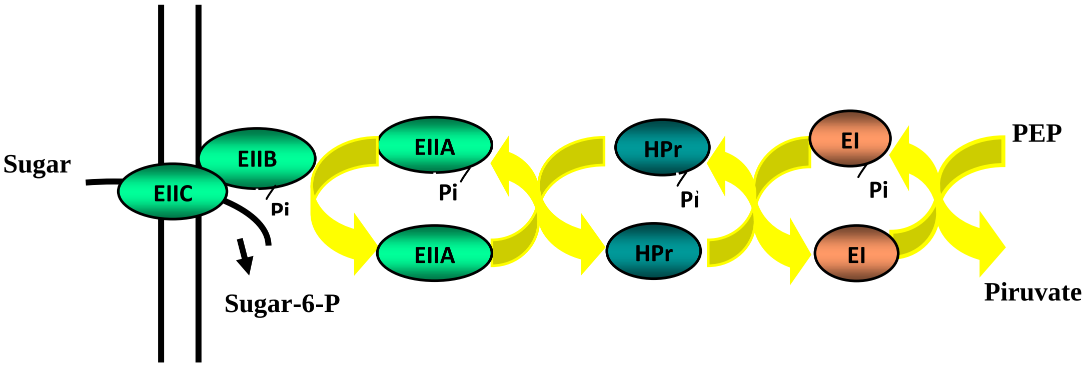

Figure 6. Representation of a typical PTS system in LAB.

Based on the protein sequence alignments of the EII components, the PTS of Gram-negative and Gram-positive bacteria have been clustered into 4 classes (Postma et al., 1993): (i) glucose class, e.g. EII^{glc} from *E. coli* ($EIIA^{glc}$/$EIICB^{glc}$) or *B. subtilis* ($EIICBA^{glc}$); (ii) mannitol class, e.g. PTS^{mtl} from *E. coli* ($EIICBA^{mtl}$), while in Gram-positive, the EIIA component is an independent protein; (iii) lactose class, e.g. PTS^{lac} from *L. casei* or *L. lactis* ($EIIA^{lac}$/$EIICB^{lac}$); (iv) mannose class, that has the particularity of possessing a EIID component, e.g. PTS^{man} from *E. coli* ($EIIAB^{man}$/$EIIC^{man}$/$EIID^{man}$) or PTS^{lev} from *B. subtilis* ($EIIA^{lev}$/$EIIB^{lev}$/$EIIC^{lev}$/$EIID^{lev}$), which corresponds to a specific PTS system for mannose and fructose also called levulose. Furthermore, regarding the EIIC phylogeny, the PTS permeases are classified in 7 families: glucose, mannitol-fructose, lactose, galactitol, glucitol or sorbitol, mannose and L-ascorbate (Barabote and Saier, 2005). In the LAB group the PTS system for transport of glucose is the most common, although the other permease families are represented (Barabote and Saier, 2005). Nevertheless, in some bacteria like *L. casei* the transport of glucose occurs preferentially by the mannose class PTS system (Yebra et al., 2006).

In addition to their role in sugar transport, the PTS systems play an important role in cellular homeostasis allowing the bacterial cells to adjust their biochemical and physiological processes according to the energy and carbon sources available (Saier and Reizer, 1994; Saier et al., 1995; Deutscher et al., 2006).

The 3´, 5´-cyclic adenosine monophosphate (cAMP) is the main element of the regulatory mechanisms known as catabolic and transient repression in Gram-negative bacteria. cAMP levels can vary depending on the enzymatic activity of adenylate cyclase, which is activated by the phosphorylated form of $EIIA^{glc}$. Thus, the variation of cAMP levels can affect its linkage to the transcriptional regulator CRP (catabolic repression protein), which controls the expression of the appropriate catabolite operons (Postma et al., 1993; Zhou et al., 2013).

By contrast, in BAL as well as in others Gram-positive bacteria with low G+C content, the phospho-carrier HPr protein is the main element in the catabolic regulatory processes. HPr is able to be phosphorylated on His-15 by transfer from EI (P-His-HPr) (Fig. 6), participating in the carbohydrate transport by the PTS systems, and can also transfer its phosphate to other non-PTS proteins, regulating their functions (Stülke et al., 1998; Deutscher et al., 2002). In addition, HPr can be phosphorylated or dephosphorylated on Ser-46 (P-Ser-HPr) by the bifunctional enzyme HPr kinase/phosphorylase (HprK/P) (Deutscher, 2008; Görke and Stülke, 2008), taking part in the regulatory mechanism of sugar uptake known as carbon catabolite repression (CCR), mainly produces in the presence of glucose. The uptake of glucose increases the concentration of the intermediate glycolytic metabolite fructose-1,6-bisphosphate and the ATP, which triggers the kinase activity of HprK/P, catalysing the formation of P-Ser-HPr. By contrast, under nutrient limitation conditions, inorganic phosphate is accumulated promotes the phosphorylase activity of HprK/P (Deutscher, 2008). P-Ser-HPr binds to the major global transcription regulator of CCR, the catabolite control protein A (CcpA). This union provokes a slight rotational movement into the CcpA core (Schumacher et al., 2004). This conformational change promotes the binding of the HPr-CcpA complex to a consensus sequence denominated catabolite repression element (*cre*) located in the promoter regions of genes and operons subject to CCR.

2.2.1.1. PTS systems for the use of sorbitol in LAB

Sorbitol, also known as glucitol, is present in many fruits and is widely used in the food industry as a sweetener, moisturizer and stabilizer (Silveira and Jonas, 2002). Its prebiotic effect has been also demonstrated, so it could be used for the manufacture of functional foods (Sarmiento-Rubiano et al., 2007).

Sorbitol as a carbon source has been reported in some bacteria like *E. coli* (Yamada and Saier, 1988), *Erwinia amylovora* (Aldridge et al., 1997), *Clostridium beijerinckii* (Tangney et al., 1998), *Streptococcus mutans* (Boyd et al., 2000) and *L. casei* (Yebra and Pérez-Martínez, 2002). In all cases, the sorbitol transport is by the PTS system.

EII proteins responsible for the sorbitol uptake (EII^{gut}) have an unusual structure, consisting in EIIA, EIIBC and EIIC, where EIIC corresponds only to the N-terminal region of the domain and the C-terminal region is fused to the EIIB domain (Aldridge et al., 1997; Tangney et al., 1998; Boyd et al., 2000; Alcántara et al., 2008).

The enzymes involved in the metabolism and transport of sorbitol are clustered and expressed in the *gut* operon. This operon includes the gene *gutF* that encodes the sorbitol-6-P dehydrogenase, which transforms sorbitol-6-P into fructose-6-P; the genes *gutR* and *gutM* that encode regulatory proteins; and the genes *gutC*, *gutB* and *gutA* that encode for the EII components of PTS^{gut}. This organization is conserved in the phylum *Firmicutes*, with a few exceptions like *C. beijerinckii* that lacks the gene *gutF* and the genus *Listeria* which lacks the gene *gutR* (Tangney et al., 1998).

The role of GutM and GutR proteins has been studied in *E. coli*, where a complex and not entirely clear mechanism has been proposed in which GutM is an activator and GutR a repressor (Yamada and Saier, 1988). In the *Firmicutes* group, the analysed *gut* operons contain homologues to the *gutM* gene, but the GutR regulator is different from that of *E. coli*.

It has been demonstrated that the GutR protein of *L. casei* is a regulator that acts as a transcriptional activator by binding to its operator upstream of the *gut* operon (Stülke et al., 1998; Alcántara et al., 2008). GutR has a PTS regulation domain (PRD) phosphorylatable by the components of the PTS system, and both the GutR operator and the PDR are conserved in *Firmicutes*. The *gutM* gene encodes for a conserved protein of unknown function in this group of bacteria, but studies performed with *L. casei* indicate that GutM plays a regulatory role (Alcántara et al., 2008).

2.3. Exopolysaccharides produced by LAB

Some LAB as well as other bacteria, fungi or algae are able to produce different types of exopolysaccharides (EPS). Generally, bacterial EPS are heterogeneous long-chain polymers of high molecular weight, which are released into the environment (Sutherland, 1972). They can be excreted outside the cell wall or be attached to it, forming a capsular layer (Ruas-Madiedo and de los Reyes-Gavilan, 2005; Chapot-Chartier et al., 2011). In general, EPS-producing bacteria do not use these polymers as an energy source. The functional role of these EPS is not completely understood, but organisms invest a great deal of energy in their production, so presumably they must play an important role for bacteria. Indeed, it has been shown that EPS influence the way in which microorganisms interact with their external environment (Donot et al., 2012). EPS are capable of retaining water, protecting bacteria from desiccation. They also act as a physical barrier and can protect bacteria from bacteriophage attack, predation by protozoans, penetration of toxic compounds, surfactants and antibiotics, as well as a defence against osmotic stress (Donot et al., 2012; Ruas-Madiedo, 2014). In addition, when EPS are secreted they are able to aggregate forming complex structures, as well as to interact with other biomolecules, participating in interactomic processes such as bacteria-eukaryotic cell and bacteria-bacteria (Ruas-Madiedo et al., 2008). Thus, they play an important role in the colonization of different ecosystems through adhesion to surfaces and the formation of biofilms, which in turn, help them to adapt to different physico-chemical conditions of the environment (Donot et al., 2012). Apart from its adhesive and protective functions, the formation of a biofilm promotes the association of different microbial species, and these in turn can create attachment sites for other species, becoming a symbiotic ecosystem where the metabolic products of one species can act as substrates for metabolic processes of another species (Sutherland, 2001; Dunne, 2002; Tytgat et al., 2018).

2.3.1. Classification

EPS produced by LAB are linear or branched biopolymers with repeating units of sugar or sugar derivatives, which are linked by glycosidic bonds. These compounds reach a very high molecular mass, even exceeding 10^8 Da (Zarour et al., 2017), depending on the number of residues that constitute their structure. Thus, LAB are able to produce a wide range of EPS that can differ in: (i) monosaccharide composition and type of linkages; (ii) primary structure with or without different degrees and type of branching; (iii) molecular weight and (iv) overall structural conformation (Ruas-Madiedo and de los Reyes-Gavilan, 2005; Finore et al., 2014). Therefore, EPS are commonly classified, based on their monosaccharide composition and biosynthetic pathway, in homopolysaccharides (HoPS) and heteropolysaccharides (HePS).

2.3.1.1. Heteropolysaccharides

HePS are composed of repeating units of at least two or more different monosaccharides, mainly glucose, galactose and rhamnose, but also can contain with less frequency N-acetylated monosaccharides (N-acetyl-glucosamine and N-acetyl-galactosamine), other monosaccharides like fucose, mannose or ribose, as well as organic and inorganic (glucuronic acid, acetyl groups, glycerol, phosphate, etc.) substituents (Torino et al., 2015; Salazar et al., 2016). In addition, the repeating unit backbone can present side groups, and all the monomers can be linked by many different types of ‘-osidic’ bonds. The use of nuclear magnetic resonance (NMR) has shown the great variety of repeating units, ranging from three to eight monomers, present in HePS synthesized by LAB, which has resulted in the description of up to 45 NMR structures (De Vuyst et al., 2001; Laws et al., 2001; Broadbent et al., 2003; Ruas-Madiedo et al., 2009). This high complexity is also detected in the biosynthesis and secretion pathways, which require the joint action of several proteins usually encoded by genes located in operons. Often, these operons harbour genes encoding proteins predicted to be involved in regulation, glycosyltransferases for the synthesis of the repeating units, as well as proteins for chain length determination, polymerization, and export (Ruas-Madiedo, 2014).

Among the variety of HePS, three of them are notable for their industrial application, kefiran, gellan gum and xanthan gum. Kefiran is a HePS produced by LAB, mainly by *Lactobacillus kefiranofaciens*, although other species of *Lactobacillus* (*L. kefirgranum*, *L. parakefir*, *L. kefir* and *L. delbrueckii* ssp. *bulgaricus*) are able to produce it (Patel et al., 2012). Kefiran consists of almost equal amounts of glucose and galactose, forming a repeating pentasaccharide unit that displays one to two monosaccharide branches in random positions (Exarhopoulos et al., 2018). The other two HePS are anionic and produced by non-LAB bacterial strains. Gellan gum is produced by the bacteria *Spingomonas paucimobilis* and *Azotobacter chroococcum* (Sutherland and Kennedy, 1996). This HePS is composed of repeating tetrasaccharide units of two residues of β-D-glucose, one of β-D-glucuronate and one of α-L-rhamnose (Zhu et al., 2013). Xanthan gum is synthesised by *Xanthomonas campestris* (Vorholter et al., 2008). This HePS is made up of pentasaccharide subunits, forming a cellulose backbone with trisaccharide side chains composed of mannose β-(1,4)-glucuronic-acid, β-(1,2)-mannose attached to alternate glucose residues in the backbone by α-(1,3) linkages (Jansson et al., 1975).

2.3.1.2. Homopolysaccharides

HoPS are composed of only one type of monosaccharide sub-unit: glucose (glucans), fructose (fructans) or galactose (polygalactans) and only one protein is responsible for the synthesis and the extracellular location of the HoPS. These glucans, fructans and polygalactans are subdivided based on the glycosidic linkage of the repeating unit backbone and the position of the carbon involved in the bond. Thus, glucans are sub-classified into α-glucans (dextran, mutan, alternan and reuteran) and β-glucans; whereas, fructans are sub-classified into levan-type and inulin-type, both being β-fructans (Torino et al., 2015; Zannini et al., 2016).

Dextran is a α-glucan with a main backbone chain linked by α-(1,6) glycosidic bonds, and may also be branched through various secondary linkages such as α-(1,2), α-(1,3) or α-(1,4). Dextran was first described in *Leuconotoc mesenteroides*, and the dextran synthesized by the dextransucrase from the NRRL B-512F strain, which consists of a dextran with 5 % of branching in α-(1,3), was one of the first biopolymers produced at industrial scale for medical and biotechnological applications (Barker et al., 1993;

Monchois et al., 1997). Furthermore, this HoPS is widely produced among LAB and it has been reported in dextran-producing species from *Weissella, Streptococcus, Pediococcus, Oenococcus, Lactobacillus* and *Leuconostoc* genera (Katina et al., 2009; Komatsu et al., 2010; Patel et al., 2010; Dimopoulou et al., 2016; Nácher-Vázquez et al., 2017b; Zarour et al., 2018).

Mutan is a α-glucan with a main backbone chain linked by α-(1, 3) glycosidic bonds, and can be branched with α-(1, 6)-linked side chains. This HoPS is produced by a mutansucrase and that of *S. mutans* forms the main constituent of the dental plaque conditioning layer, being this bacterium the essential virulence factor associated with biofilm development in dental caries (Hamada and Slade, 1980; Koo et al., 2013). Also, other strains belonging to *Leuconostoc*, *Streptococcus* and *Lactobacillus* genera are able to produce mutan (Côté and Skory, 2012; Côté et al., 2013).

Alternan is an α-glucan whose backbone chain consists of alternating α-(1,6) and α-(1,3) glycosidic linkages that can have some degree of α-(1,3) branching (Argüello-Morales et al., 2000). This unique structure confers to this HoPS physical characteristic such as high solubility, low viscosity and resistance to bacterial and enzymatic hydrolysis (Smith et al., 1994; Leathers et al., 2003). The first instance of alternan was its production by *L. mesenteroides* NRRL B-1355 strain (Côté and Robyt, 1982), synthesised by the glucansucrase ASR (also known as alternansucrase) that is unable to synthesize two consecutive α-(1,3) linkages (Côté and Sheng, 2006).

Reuteran, as the name suggests, is a specific α-glucan synthesised by *L. reuteri*. Reuteran-producing *L. reuteri* 121 was the first know strain that synthesised the HoPS by action of the glucansucrase GTFA (reuteransucrase), and contains in large amount α-(1,4) linkages (58%) and α-(1,6) linkages (42%) with apparently no repeating units (Kralj et al., 2002). The structure of the reuteran produced by GTFA was elucidated in alternating α-(1, 4) and α-(1, 6) linkages with no consecutive α-(1, 6) detected (Kralj et al., 2002; Kralj et al., 2004). However, another reuteransucrase from *L. reuteri* ATCC 55730, named GTFO, was found to produce a higher proportion of α-(1,4) linkages (80%) and less α-(1,6) linkages (20%) in its polymer, with no alternating structure (Kralj et al., 2005).

The β-glucan produced by LAB has a backbone of β-(1, 3)-linked units, branched with β-(1, 2)-linked side chains of varying distribution. The production of β-glucan by LAB is uncommon, and it has been described for only some LAB isolated from alcoholic fermented beverages: *Pediococcus parvulus* IOEB8801 and *Oenococcus oeni* IOEB0205 (Llaubères et al., 1990; Dols-Lafargue et al., 2008) strains isolated from wine; *P. parvulus* 2.6, CUPV1 and CUPV22, *Lactobacillus suebicus* CUPV221, *Lactobacillus diolivorans* G77 and *O. oeni* I4 (Dueñas-Chasco et al., 1997; Dueñas-Chasco et al., 1998; Ibarburu et al., 2007; Garai-Ibabe et al., 2010). Also it has been reported that several strains of the non-LAB probiotic bacteria *Propionibacterium freundenreichii* are able to produce this kind of β-glucan (Nordmark et al., 2005; Deutsch et al., 2010). β-glucans are synthesised by a glycosyltransferase, a transmembrane enzyme that utilizes UDP-glucose as donor substrate (Werning et al., 2006). Finally, the pathogen LAB *Streptococcus pneumoniae* serotype 37 forms a capsule with a β-glucan similar to that of the other LAB (Adeyeye et al., 1988),

Fructans are HoPS made of β-D-fructosyl molecules that can be linked by β-(2, 1) or β-(2, 6) bonds, forming inulins or levans respectively. Inulin-type EPS are synthesised from sucrose by an inulosucrase enzyme, and they have been describe in LAB such as *Lactobacillus johnsonii* NCC 533, *S. mutans* JC2 and *L. reuteri* 121 (Anwar et al., 2008; Patel et al., 2012). In turn, levan EPS are produced by a levansucrase and have been described in LAB such as *Lactobacillus sanfranciscencis* LTH 2590, *L. reuteri* LB121 and *L. mesenteroides* NRRL B-512 F (Patel et al., 2012). All of them are considered prebiotic compounds.

Polygalactans are uncommon HoPS composed of a pentameric repeating unit of galactose, whose main chain consists of three galactose molecules linked by β-(1,3) and β-(1,4) bonds and has a side chain of two galactoses linked by β-(1,3) bonds. They were described for the strain *L. lactis* sbsp. *cremoris* H414 (Gruter et al., 1992).

2.4 Pediococcus parvulus 2.6

This bacterium was isolated from natural Basque cider, in which it is responsible for an unwanted alteration called ‘ropiness’ or ‘oiliness’ which is due to the production of an EPS (Fernández et al., 1995). The presence of EPS provided a raised phenotype in the bacterial colonies when grown on solid media, as well as a viscous aspect in the growth in liquid medium. At first, the strain was described as *Pediococcus damnosus* 2.6, but later using 16s rDNA sequencing techniques it was definitively given the name *P. parvulus* 2.6 (Werning et al., 2006). As previously mentioned, this strain produces a β-glucan and its physical-chemical characterization showed that the structure was formed by a main chain of glucopyranose molecules linked by β-(1,3) bounds, which branches in the *O*-2 position of one of every two residues, formed by a single molecule of β-glucopyranose (Dueñas-Chasco et al., 1997). The gene *gtf* encodes the GTF glycosyltransferase that belongs to the COG1215 membrane-bound glycosyltransferase family and consists in a cytosolic glycosyltransferase domain flanked by two and four transmembrane segments (Werning et al., 2006). This is the only enzyme involved in the synthesis of this β-glucan (Werning et al., 2008). The gene *gtf* is located in the natural plasmid pPP2, and an isogenic EPS non-producing 2.6NR strain was obtained by curing this plasmid (Fernández et al., 1995). *P. parvulus* 2.6, was able to resist *in vitro* simulated gastrointestinal stress and adhered to enterocytes in an *in vitro* Caco-2 cell model (Fernández de Palencia et al., 2009). Also, the capacity of its EPS to modulate human macrophages cytokine profile has been investigated (Notararigo et al., 2014).

In this work, I have extended the study of the probiotic properties and immunomodulatory capacities of this bacterium and its β-glucan. I have investigated the bacterium's metabolic capacities with emphasis on its sorbitol utilisation and its relationship with the EPS production, I have used zebrafish as *in vivo* model to evaluate their properties and I have investigated the use of the bacterium to produce functional foods enriched with the bacterial β-glucan.

Objectives

The eukaryotic (1, 3)-β-glucans are well-known immunomodulators, and the bacterial 2-substituted (1, 3)-β-glucan produced by LAB has been suggested as a potential immunomodulator. *P. parvulus* 2.6 is the most characterized bacterium, which produces this bacterial β-glucan.

Thus, the aim of this thesis was to obtain in depth knowledge of the genetic and functional characteristics of *P. parvulus* 2.6 and the production of its β-glucan, as well as to gain knowledge in the immunomodulatory properties of this biopolymer. To achieve these goals, the next objectives were proposed:

1. The development and/or usage of tools for:
    1.1. The detection and quantification of the β-glucan produced by *P. parvulus* 2.6.
    1.2. The detection of lactic acid bacteria and the study of gene expression by the usage of the mCherry fluorescent protein.
2. To investigate the sorbitol metabolism and its relationship with the β-glucan biosynthesis in *P. parvulus* 2.6. Furthermore, the sorbitol utilization will be studied at the molecular level.
3. The fluorescent labelling of *P. parvulus* 2.6 for the detection of its presence during the evaluation of its probiotic properties.
4. The evaluation of the probiotic properties of *P. parvulus* 2.6 using an *in vitro* Caco-2 cells model and an *in vivo* gnotobiotic zebrafish larvae model.
5. The *in vivo* evaluation of the immunomodulation properties of the pediococcal β-glucan using a gnotobiotic and an inflammation induced zebrafish models.
6. The development and evaluation of new cereal-based foods by fermentation with *P. parvulus*.

Experimental work

A specific immunological method to detect and quantify bacterial 2-substituted (1, 3)-β-D-glucan. (2014) *Carbohydrate polymers* 113:39-45

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CHAPTER 1

Resumen

Los exopolisacáridos (EPS) de las bacterias del ácido láctico (BAL) han sido y están siendo ampliamente estudiados debido a su gran interés, tanto a nivel industrial, al ser utilizados para la mejora de las propiedades organolépticas de los alimentos, como por sus efectos beneficiosos para la salud al actuar como compuestos prebióticos sobre la microbiota del tracto digestivo y como moduladores del sistema inmune (Pérez-Ramos et al., 2016). Así, durante los últimos años se han ido describiendo nuevas bacterias productoras de EPS, entre ellas *Pediococcus parvulus* 2.6 aislada de sidra y objeto de estudio de este trabajo. Su EPS es un homopolisacárido con una cadena principal, constituida por moléculas de glucopiranosa unidas por enlaces β-(1,3), que se ramifica en la posición *O*-2 de uno de cada dos residuos, con cadenas laterales formadas por una única molécula de β-glucopiranosa. Este EPS (β-glucano) ha sido descrito en un número reducido de BAL aisladas de bebidas alcohólicas. Nuestro grupo ha mostrado que este β-glucano posee propiedades prebióticas e inmunomoduladoras (Russo et al, 2012; Notararigo et al., 2014) y por tanto la detección de nuevas bacterias productoras de este tipo de EPS es de gran interés dentro del campo de la alimentación funcional. Sin embargo, los métodos habitualmente utilizados para la detección y cuantificación de los EPS, como el método colorimétrico del fenol-sulfúrico (DuBois et al., 1956), requieren la purificación previa de los polímeros para eliminar los posibles carbohidratos contaminantes presentes en las muestras, y una posterior caracterización fisicoquímica para obtener su estructura. Así, el desarrollo de un método específico para la detección del β-glucano en el sobrenadante de los cultivos bacterianos permitiría una rápida identificación de las BAL productoras y una cuantificación directa de los niveles de producción.

La enzima responsable de sintetizar el β-glucano es la GTF glicosiltransferasa (GTF). Este hecho fue demostrado por nuestro grupo al clonar el gen *gtf*, que codifica la GTF de *P. parvulus* 2.6, en el plásmido pNGTF y expresar la enzima en forma activa en *Lactococcus lactis* NZ9000, con una síntesis concomitante de un β-glucano con la misma estructura que el EPS pediocócico (Werning et al., 2008). La GTF de *P. parvulus* 2.6 posee una similitud del 33% a nivel de aminoácidos con la Tts glicosiltransferasa de *Streptococcus pneumoniae* serotipo 37, responsable de la síntesis de un polisacárido capsular muy similar en estructura al EPS de *Pediococcus*. Además, nuestro grupo de investigación demostró previamente, que un anticuerpo desarrollado frente a la cápsula de *S. pneumoniae* serotipo 37 (Ac anti-37) era capaz de aglutinar a la bacteria *P. parvulus* 2.6 y no a la cepa isogénica *P. parvulus* 2.6NR, que no produce el β-glucano (Fernández de Palencia et al., 2009). Con estos antecedentes, en este trabajo nos planteamos diseñar y validar un ensayo de inmunoabsorción ligado a enzimas (ELISA) de inhibición basado en la utilización del Ac anti-37 como anticuerpo primario para la detección y cuantificación rápida del β-glucano, así como para el estudio bioquímico de la actividad enzimática de las GTF glicosiltransferasas que lo producen.

Así, el β-glucano producido por *L. lactis* NZ9000[pNGTF] y posteriormente purificado, fue utilizado para el desarrollo del método ELISA de inhibición. Esquemáticamente, el inmunoensayo consiste en inmovilizar el β-glucano purificado (62,5 ng mL^{-1}) en los pocillos de una placa de microtítulo a 4 ºC durante 16 h. Independientemente, las muestras a analizar se exponen al Ac anti-37 (diluido 1:800) durante 30 min a temperatura ambiente y posteriormente las mezclas se adicionan a los pocillos de la placa y se incuban durante 1 h y 30 min a temperatura ambiente. Después de lavar los pocillos, el Ac anti-37 unido al β-glucano inmovilizado en los pocillos se conjuga con un anticuerpo policlonal (diluido 1:25.000) ligado a la fosfatasa alcalina, durante 2 h a temperatura ambiente. Por último, se adiciona una solución que contiene *p*-nitrofenilfosfato y se incuba la placa en oscuridad durante 30 min a 37 ºC. La actividad de la fosfatasa alcalina hidroliza el *p*-nitrofenilfosfato liberando fosfato inorgánico y la base conjugada *p*-nitrofenol que es un compuesto de color amarillo, que se detecta por absorbancia a 415 nm en un espectrofotómetro. Para correlacionar los valores de absorbancia con la concentración del β-glucano se genera una recta patrón utilizando, como EPS competidor unido al Ac anti-37, concentraciones conocidas del β-glucano purificado. Los resultados obtenidos revelaron que existe una relación lineal entre los incrementos de absorbancia y los de concentración del EPS en un rango entre 20 ng mL^{-1} y 2500 ng mL^{-1}. Para determinar la especificidad del método se utilizaron soluciones de otros polímeros: un (1,3)-β-glucano lineal (curdlano), un (1,3)-β-glucano ramificado en posición *O*-6 (laminarina) y un heteropolisacarido ramificado (xantano). Ni el xantano ni el curdlano fueron capaces de competir con el β-glucano inmovilizado por su unión al Ac anti-37. Sin embargo, la laminarina si fue capaz de competir, pero a unas concentraciones muy elevadas, mostrando una correlación lineal entre 10 µg mL^{-1} y 250 µg mL^{-1}.

Otra de las aportaciones de este trabajo fue la estandarización de la detección y cuantificación del β-glucano directamente en los sobrenadantes de cultivos bacterianos mediante el inmunoensayo. El método fue validado con sobrenadantes de cultivos de bacterias productoras del β-glucano aisladas de sidra (*P. parvulus* 2.6, *Oenococcus oeni* I4, *Lactobacillus diolivorans* G-77) y de la estirpe recombinante *L. lactis* NZ9000[pNGTF], crecidos en medios complejos (MRS y M17), que contienen polisacáridos. Además, la utilización de este método reveló valores basales muy bajos en las muestras controles, correspondientes a los medios de cultivo y los sobrenadantes de estirpes no productoras.

Por otra parte, se evaluó comparativamente la eficacia del inmunoensayo, que cuantifica el β-glucano directamente en el sobrenadante de los cultivos bacterianos, con el método clásico colorimétrico del fenol-sulfúrico, que valora el contenido total de carbohidratos neutros en la muestra y requiere una precipitación previa de los EPS con etanol. Se crecieron las bacterias productoras anteriormente citadas en medios definidos que no contienen otros polisacáridos, y se analizaron sus sobrenadantes utilizando ambos métodos. La cuantificación por el método ELISA de inhibición reveló valores de concentración de β-glucano entorno a un 36-61 % superior a los detectados con el método del fenol-sulfúrico en los sobrenadantes de todas las estirpes analizadas. Estas diferencias podrían deberse a que moléculas del EPS de bajo peso molecular no precipitaran con etanol. Para probar esta hipótesis, se cuantificó la concentración de β-glucano antes y después de su purificación por precipitación con etanol utilizando el método ELISA, y como se esperaba se comprobó que en este proceso de purificación se produce una reducción de los niveles iniciales, en torno al 20-38 %.

Independientemente, cabe destacar que durante el desarrollo de esta tesis hemos utilizado el imunoensayo para valorar el β-glucano en los sobrenadantes de cultivos bacterianos (ver detalles en el Capítulo 4), así como unido a *P. parvulus* 2.6 (ver detalles en el Capítulo 5) y validado su utilización para realizar una cuantificación específica de este EPS en matrices alimentarias (ver detalles en el Capítulo 6).

Finalmente, el método ELISA de inhibición fue utilizado para caracterizar bioquímicamente la actividad enzimática de la GTF, en lugar de emplear el método estándar que requiere el uso del sustrato radioactivo UDP-[^{14}C]glucosa. Para los ensayos se utilizaron vesículas membranosas de *L. lactis* NZ9000[pNGTF], que contenían la enzima GTF y como sustrato la UDP-glucosa. Se determinó que la constante de afinidad (K_m) de la enzima era 123 ± 3 µM y la velocidad máxima (V_{max}) de catálisis era 18 ± 0,8 ng de EPS por mg de proteína total por minuto. La K_m obtenida in *vitro* para la GTF glicosiltransferasa fue similar a las reportadas para otras enzimas de su misma familia (Werning et al., 2006; Werning et al., 2008).

Mi aportación a este trabajo

Para lograr la consecución de este trabajo científico, mi aportación fue la siguiente. Contribuí al desarrollo y validación del método ELISA de inhibición, así como a la detección y cuantificación del β-glucano en los sobrenadantes de los cultivos de las estirpes productoras. Finalmente, también participé en la elaboración de la Tabla 1 y de la Figura 4 del trabajo.

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A specific immunological method to detect and quantify bacterial 2-substituted (1,3)-β-D-glucan

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ABSTRACT

Exopolysaccharides synthesized by lactic acid bacteria have prebiotic properties and contribute to the rheology and texture of fermented foods. Here, we have standardized an immunological method for the specific detection of 2-substituted (1,3)-β-D-glucans. The method allows direct detection and quantification of this exopolysaccharide in culture supernatants containing other mono- and poly-saccharides. Moreover, it allows specific detection of the biomolecules synthesized *in vitro* in enzymatic reactions. Thus, this method allows the fast identification of producing bacteria, as well as biochemical characterization of the glycosyltransferases responsible for their synthesis.

1. Introduction

Most commercialized probiotic microorganisms are lactic acid bacteria (LAB) and some produce exopolysaccharides (EPS) (Ruas-Madiedo, Abraham, Mozzi, & de los Reyes-Gavilán, 2008; Salminen et al., 1998; Ventura, Canchaya, Fitzgerald, Gupta, & van Sinderen, 2007). In addition, some LAB strains belonging to the *Pediococcus*, *Lactobacillus* and *Oenococcus* genera, isolated from cider and wine, are able to produce a 2-substituted (1,3)-β-D-glucan EPS (Dols-Lafargue et al., 2008; Dueñas-Chasco et al., 1997, 1998; Ibarburu et al., 2007; Llaubères, Richard, Lonvaud, Dubourdieu, & Fournet, 1990). Analysis of the rheological properties of the 2-substituted (1,3)-β-D-glucan synthesized by *Pediococcus parvulus* 2.6R showed that it has potential utility as a biothickener (Lambo-Fodje et al., 2007; Velasco et al., 2009). *Pediococcus* (Fernández de Palencia et al., 2009) and *Lactobacillus* (Garai-Ibabe et al., 2010) strains that produce this EPS have increased adherence to Caco-2 cells. *P. parvulus* 2.6R is able to immunomodulate macrophages (Fernández de Palencia et al., 2009) and production of the 2-substituted (1,3)-β-D-glucan confers to the intestinal *Lactobacillus paracasei* NFBC 338 higher resistance to gastrointestinal and technological stresses (Stack et al., 2010). In addition, human consumption of oat-based food prepared with *P. parvulus* 2.6R resulted in a decrease of serum cholesterol levels, boosting the effect previously demonstrated for (1,3)(1,4)-β-D-glucans of oat-based products (Mårtensson et al., 2005). Moreover, the production of yogurt and various beverages with 2-substituted (1,3)-β-D-glucan producing LAB indicates the advantageous techno-functional properties of these strains (Elizaquível et al., 2011; Kearney et al., 2011). Furthermore, the purified EPS from *P. parvulus* 2.6R increased the growth, viability and adherence capability of probiotic LAB (Russo et al., 2012). Therefore, LAB producing this EPS could have potential as probiotic strains and the 2-substituted (1,3)-β-D-glucan itself appears to have prebiotic properties.

The specific detection and quantification of EPS produced by LAB, especially in a background that contains other carbohydrates, is laborious and time-consuming. The colorimetric phenol–sulphuric method (Dubois, Gilles, Hamilton, Rebers, & Smith, 1956) to quantify the total polysaccharide content of samples, requires the removal of any disaccharides or monosaccharides present in the media by ultrafiltration, dialysis or ethanol precipitation (Ruas-Madiedo & de los Reyes-Gavilán, 2005; Notararigo et al., 2013). A complete characterization of the polysaccharide

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Abbreviations: DM, defined medium; ELISA, enzyme linked immunosorbent assay; EPS, exopolysaccharide; GLC, gas–liquid chromatography; GLC–MS, gas–liquid chromatography–mass spectrometry; HP-SEC, high-performance size exclusion chromatography; LAB, lactic acid bacteria; PBS, Phosphate buffered saline; SDM, semi-defined medium.

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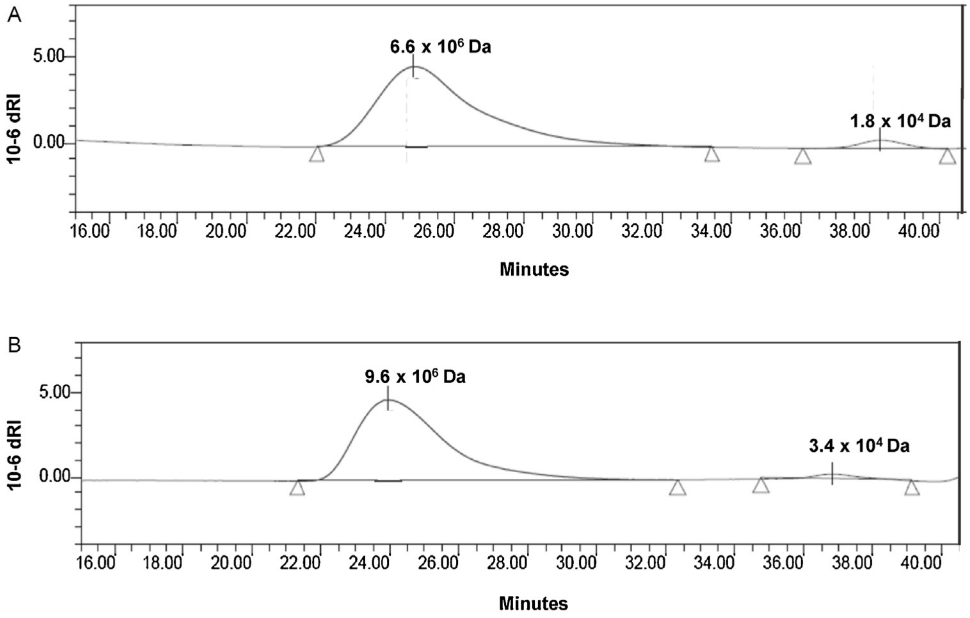

Fig. 1. Analysis of purified EPS produced by *L. lactis* NZ9000[pNGTF] (A) and *P. parvulus* 2.6R (B) by high-performance size exclusion chromatography using as molecular weight standards dextrans of 10^4, 4×10^4, 7×10^4, 1.5×10^5, 2.7×10^5, 4.1×10^5, 6.7×10^5, 1.4×10^6 and 2×10^6 Da (Fluka).

then usually requires further purification, followed by relatively sophisticated, derivatization, degradation and analytical techniques. Therefore a simple direct method for the specific and quantitative detection of 2-substituted (1,3)-β-D-glucan synthesized by bacteria would facilitate the identification of new potential probiotic strains, and the quantification of 2-substituted (1,3)-β-D-glucan production.

LAB 2-substituted (1,3)-β-D-glucans are synthesized by GTF glycosyltransferase (Werning et al., 2006, 2008), which has significant identity (33%) only with the Tts glycosyltransferase of *Streptococcus pneumoniae* serotype 37 (Llull, Muñoz, López, & García, 1999). This latter enzyme synthesizes this organism's capsule (Llull, García, & López, 2001), which is a β-D-glucan similar to that synthesized by *Pediococcus* and *Lactobacillus* strains. Anti-serotype 37 antibodies also agglutinate *Lactococcus lactis* and *Lb. paracasei* NFBC 338 strains that over-produce GTF glycosyltransferase (Dols-Lafargue et al., 2008; Stack et al., 2010; Werning et al., 2008) as well as LAB strains naturally carrying this gene (Dols-Lafargue et al., 2008; Werning et al., 2006). Therefore, in this work, we report on the design and validation of a competition enzyme linked immuno-sorbent assay (ELISA) method for the specific detection of 2-substituted (1,3)-β-glucans, based on *S. pneumoniae* serotype 37 antibodies.

2. Materials and methods

2.1. Bacterial strains and growth conditions

The following bacteria were used in this work. The 2-substituted (1,3)-β-D-glucan-producer strains isolated from Basque natural cider: *Lactobacillus diolivorans* G-77 (Dueñas-Chasco et al., 1998), *Oenococcus oeni* I4 (Ibarburu et al., 2007), *P. parvulus* 2.6R, and its isogenic non-producing strain (2.6NR) (Dueñas-Chasco et al., 1997). The 2-substituted (1,3)-β-D-glucan-producing *L. lactis* NZ9000[pNGTF] recombinant strain, which carries in the pNGTF plasmid, based on the pNZ8048 vector, the *P. parvulus* 2.6R *gft* gene under control of the PnisA promoter, and its isogenic β-D-glucan non-producing strain *L. lactis* NZ9000[pNZ8048] (Werning et al., 2008).

The *Pediococcus* strains were routinely grown at 30 °C in Man Rogosa Sharpe (MRS) broth (De Man, Rogosa, & Sharpe, 1960) supplemented with 0.5% glucose and 0.1% Tween 80. *O. oeni* I4 was routinely grown at 30 °C under anaerobic conditions in MRS supplemented with 0.5% fructose and 1% glucose. *Lb. diolivorans* G-77 was routinely grown at 30 °C in MRS supplemented with 0.5% glucose. The *Lactococcus* strains were routinely grown at 30 °C in M17 broth (Terzaghi & Sandine, 1975) supplemented with chloramphenicol (5 µg ml^{-1}).

For EPS production, batch fermentations without pH control were carried out in a semi-defined medium (SDM) supplemented with glucose (2%) (Dueñas-Chasco et al., 1997) or MRS for *P. parvulus* 2.6R and *Lb. diolivorans* G-77 as well as in the same media supplemented with fructose (1%) for *O. oeni* I4 or in a defined medium (DM) (Sánchez et al., 2008) or M17, both supplemented with glucose (0.5%) and chloramphenicol (5 µg ml^{-1}) for *L. lactis* NZ9000[pNGTF]. *Pediococcus*, *Lactobacillus* and *Oenococus* strains were grown to stationary phase to increase production of the EPS. *L. lactis* strains were grown in the indicated media until OD$_{600}$ = 0.6. Then, for *L. lactis* NZ9000[pNGTF] only, expression of the *gtf* gene encoding the GTF glycosyltransferase was induced with nisin (0.25 ng ml^{-1}) 24 h prior to testing. The strains were stored at −80 °C in either MRS or M17 media supplemented with 20% (v/v) glycerol.

2.2. Isolation, purification and characterization of EPS synthesized by LAB

The 2-substituted (1,3)-β-glucan samples from *P. parvulus* 2.6R and *L. lactis* NZ9000[pNGTF] were isolated and purified from culture supernatants by ethanol precipitation and dialysis as previously described (Notararigo et al., 2013). Stock solutions of both purified EPS were prepared at 1 mg ml^{-1} in phosphate buffered saline (PBS) pH 7.0 and stored at −20 °C. For ELISA assays, EPS stock solutions were diluted in PBS pH 7.0 prior to analysis. The molecular masses of the EPS were determined by high-performance size exclusion chromatography (HP-SEC, GPCV 2000, Waters), as previously described (Velasco et al., 2009).

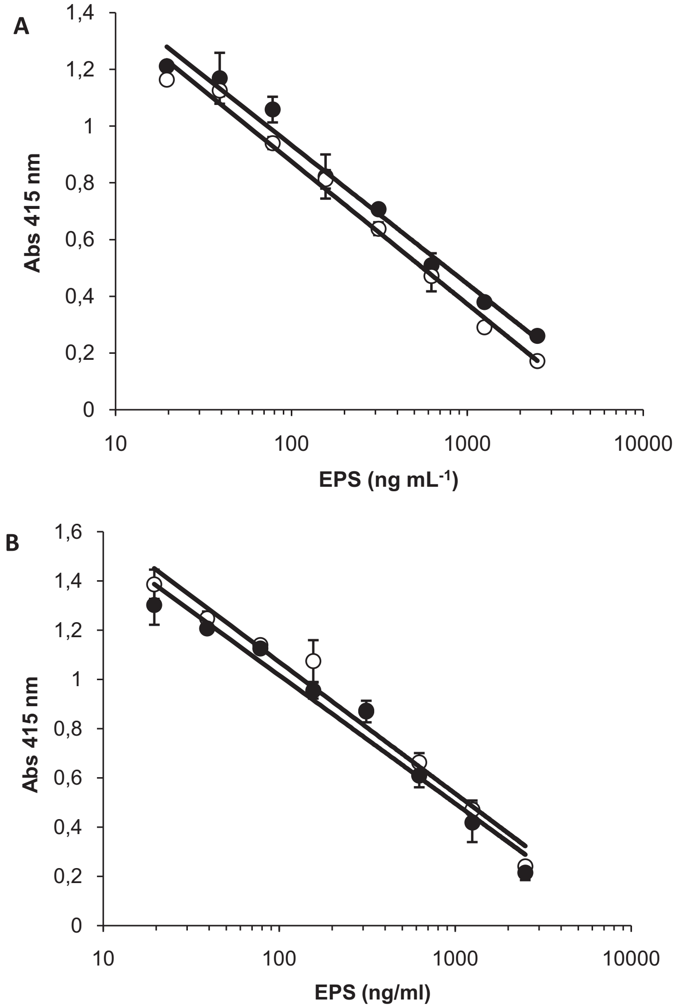

Fig. 2. Range of detection of purified 2-substituted (1,3)-β-D-glucan by the immunological method. EPS produced by *L. lactis* NZ9000[pNGTF] (A) and *P. parvulus* 2.6R (B) immobilized in microtitre plates and purified EPS from *L. lactis* NZ9000[pNGTF] (●) and *P. parvulus* 2.6R (○) were tested as competitors. The values are the mean of three independent experiments. Standard deviations are indicated as vertical bars over dots.

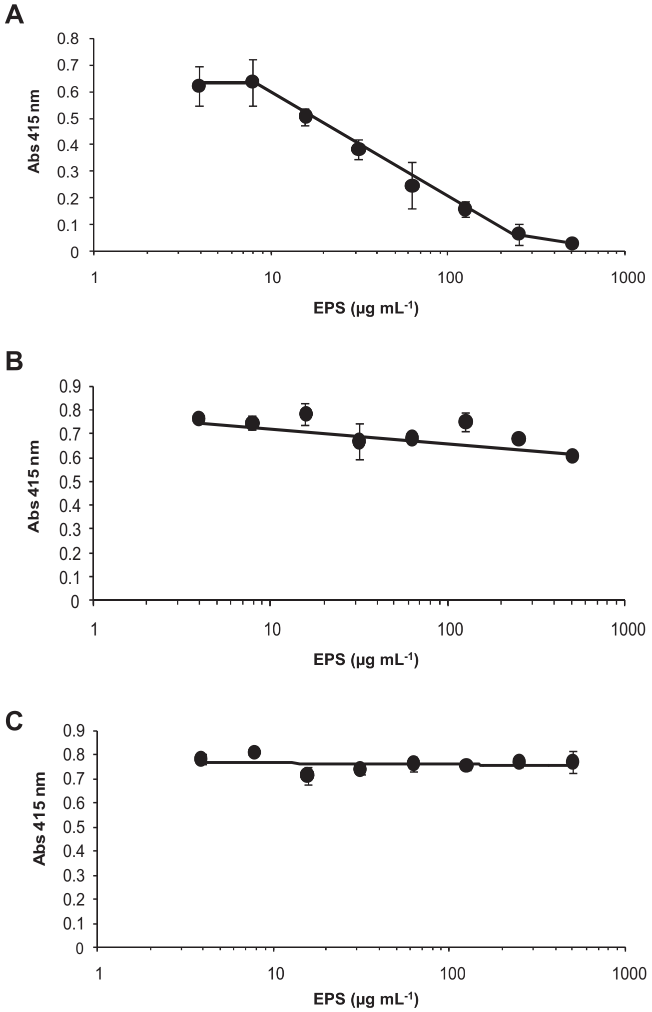

Fig. 3. Specificity of the immunological method. Laminarin (A), curdlan (B) and xanthan (C) were tested as competitors. The values are the mean of three independent experiments. Standard deviations are indicated as vertical bars over dots.

2.3. ELISA assays

ELISA assays were carried out in 96-well Nunc Maxisorp microtitre plates (Thermo Fisher Scientific, USA). The samples (cultures supernatants or purified polysaccharides) and a negative control without biopolymers were tested in duplicate in each assay. In the standard assay the 2-substituted (1,3)-β-D-glucan synthesized by *L. lactis* NZ9000[pNGTF] or by *P. parvulus* 2.6R was used at 62.5 ng ml^{-1} as immobilized EPS as well as at various concentrations as competitor for binding to the primary antibody (dilution 1:800 of anti-serotype 37, Statens Serum Institut, Denmark). In addition, to test specificity of the method, laminarin from *Laminaria digitata*; curdlan from *Alcaligenes faecalis* and xanthan from *Xanthomonas campestris* (all from Sigma-Aldrich, Germany) were used as competitors at concentrations from 3.9 µg ml^{-1} to 2.5 mg ml^{-1}.

To optimize the ELISA assay, various 2-substituted (1,3)-β-glucan solutions (31.25, 62.5, 125, 250 and 500 ng ml^{-1}) were used to coat the wells of microtitre plates (200 µl/well) by incubation for 16 h at 4 °C. The wells were washed twice with 200 µl of solution I (PBS pH 7.0 and 0.05% Tween 20), and then 300 µl of blocking solution (solution I plus 0.5% gelatin) were added, followed by 3 h of incubation at 21 °C. To perform the competition assay 150 µl of each dilution of the primary antibody (1:200, 1:400, 1:800 or 1:1600) was incubated for 30 min at 21 °C with 150 µl of each EPS dilution (from 2.4 ng ml^{-1} to 5.0 µg ml^{-1}). Then, 200 µl of the mixture were added to each activated well of the microtitre plates and after 1.5 h of incubation at 21 °C (to allow binding of free antibody to the immobilized 2-substituted (1,3)-β-glucan) the wells were washed three times with solution I. The amount of primary antibody bound to the wells was detected by addition of 200 µl of the secondary antibody (polyclonal Anti-IgG against rabbit alkaline phosphatase, Sigma-Aldrich, Germany) diluted 1:25,000 in blocking solution, incubation for 2 h at 21 °C, four washes of the wells with solution I followed by the addition of 200 µl of developing solution (*p*-nitrophenylphosphate at 1 mg ml^{-1}) in diethanolamine buffer (0.2 M diethanolamine pH 9.8 and 5 mM $MgCl_2$, Sigma) and a final incubation for 30 min at 37 °C. Then, the reactions were stopped by addition of 2 N NaOH (50 µl) and the absorbance at 415 nm was measured in a microtitre plate reader (BioRad model 680).

2.4. Phenol–sulphuric method

To determine the 2-substituted (1,3)-β-D-glucan concentration by this method, two replicates of 0.5 ml culture supernatants were precipitated overnight with 1 ml of ethanol at −20 °C and sedimented by centrifugation (13,600 × *g*, 20 min 4 °C). The

Table 1
Comparative analysis of exopolysaccharide production by ELISA and phenol–sulphuric methods.

Bacteria	Method				
	ELISA			Phenol–sulphuric	Phenol–sulfuric/ELISA
	EPS concentration				
	Total[a] (μg ml^{-1})	Precipitated[b] (μg ml^{-1})	Precipitated[b]/total[a] (%)	Precipitated[b] (μg ml^{-1})	Precipitated[b]/precipitated[b] (%)
P. parvulus 2.6R	322 ± 15	200 ± 35	62	117 ± 21	58
L. lactis NZ9000/pNGTF	235 ± 35	183 ± 19	78	117 ± 8	64
O. oeni I4	118 ± 7	94 ± 9	80	59 ± 5	63
L. diolivorans G-77	211 ± 40	166 ± 24	79	64 ± 6	39

[a] Total: concentration of the EPS was determined directly in culture supernatants.

[b] Precipitated: concentration of the EPS present in culture supernatants was determined after ethanol precipitation.

precipitates were twice resuspended in 0.5 ml of distilled water and ethanol reprecipitated and resedimented as described above. Finally, after resuspension in distilled water, the concentration of the biopolymer present in the precipitates was determined by the phenol–sulphuric method as reported by Dubois et al. (1956).

2.5. *Analysis of GTF glycosyltransferase activity*

Membrane vesicles displaying GTF glycosyltransferase activity, were prepared from *L. lactis* NZ9000[pNGTF] as previously described (Werning et al., 2008) and the enzymatic reactions were carried out as follows. Duplicate 100 μl reactions were prepared containing 12.5, 25 or 50 μg of membrane proteins as well as a range of UDP-glucose (Sigma) concentrations (from 0.04 to 25 mM), 1 mM $CaCl_2$, 9 mM $MgCl_2$ and 50 mM NaCl. These were incubated for 30 min at 30 °C then the reactions were stopped by addition of 900 μl of 100% ethanol. After 2 h incubation at 4 °C, samples were centrifuged at 13,226 × *g* at 4 °C for 20 min, the precipitates were air dried, and resuspended in 500 μl of PBS buffer pH 8.0. The reaction product was quantified by the ELISA method. The kinetic parameter, apparent K_m and apparent $V_{\max}$ were determined from Lineweaver Burk plots.

2.6. *Chemical analysis of the enzymatic reaction product*

The neutral sugar composition and linkage type was determined using polysaccharide synthesized in duplicate 1 ml reactions containing membrane vesicles (50 mg of total proteins) with or without 25 mM UDP-glucose. The ethanol precipitates, after air drying, were resuspended in 1 ml of distilled H_2O plus 5 μl of 10% SDS, incubated for 15 min at 37 °C and dialyzed (molecular mass cut-off 3.5 kDa) against distilled H_2O for 24 h. Then, 100 μl aliquots containing the membrane vesicles and the reaction product were hydrolyzed, acetylated and analyzed for the presence of neutral sugars by gas–liquid chromatography (GLC) or after methylation, hydrolysis, reduction and acetylation for the determination of the structural unit of the polysaccharide by gas–liquid chromatography–mass spectrometry (GLC–MS) as previously described (Werning et al., 2008).

3. Results

3.1. *Sensitivity of the ELISA assay*

To determine the sensitivity of the method two samples of 2-substituted (1,3)-β-glucans synthesized by either *L. lactis* NZ9000[pNGTF] or *P. parvulus* 2.6R were purified and their molecular masses determined by HP-SEC. The average molecular masses of the two main fractions were 6.6 × 10^6 and 1.8 × 10^4 Da for *L. lactis* (Fig. 1A) and of 9.6 × 10^6 and 3.4 × 10^4 Da for *P. parvulus* (Fig. 1B). The higher molecular mass fraction was the most abundant for both strains constituting, respectively, 96% and 92% of the total polymer population. Both EPS preparations were used for testing as immobilized β-glucans and as competitors for binding to the primary antibody. Both EPS preparations were immobilized efficiently (Fig. 2A *versus* B) and both purified β-D-glucans could be quantified within a range of 20 to 2500 ng ml^{-1}, independently of the origin of the immobilized polymer (Fig. 2).

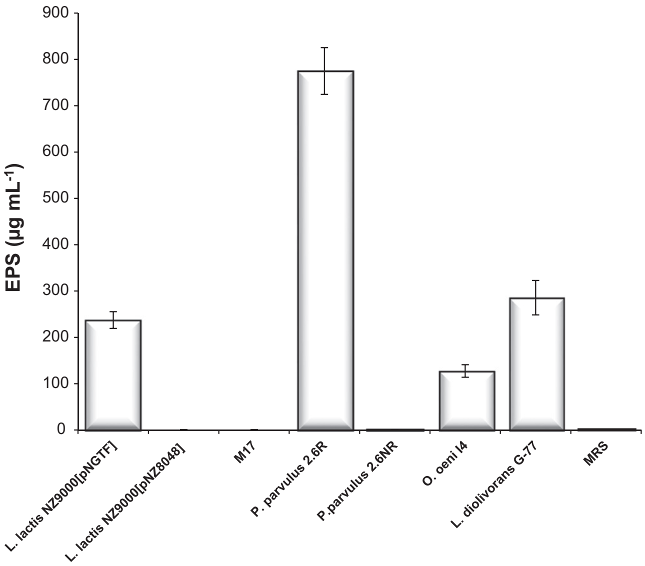

Fig. 4. Detection of 2-substituted (1,3)-β-D-glucan in culture supernatants. (A) The indicated *L. lactis* strains were grown and expression of the EPS induced in either M17 medium. *P. parvulus* 2.6R and 2.6NR, *Lb. diolivorans* G-77 and *O. oeni* I4 were grown in MRS medium. Triplicates of these cultures were then centrifuged and the supernatants were directly used for quantification of the β-D-glucan present by the ELISA assay. The values are the mean of three independent experiments. Standard deviations are indicated as vertical bars over bars.

3.2. *Specificity of the assay*

Using the purified β-D-glucan from *L. lactis* (described above) as the immobilized substrate, we tested the specificity of the method using three polysaccharides as competitors: (i) laminarin, a 6-substituted (1,3)-β-D-glucan (Fig. 3A); (ii) curdlan, a linear (1–3)-β-D-glucan (Fig. 3B) and (iii) xanthan, a branched heteropolysaccharide (Fig. 3C). Xanthan was unable to bind to the primary antibody even at very high concentration (500 μg ml^{-1}) and curdlan also showed a very low affinity for the antibody at 250 μg ml^{-1}. Laminarin was able to compete with the immobilized 2-substituted (1,3)-β-D-glucan in a linear mode, when tested in the range 10 to 250 μg ml^{-1}.

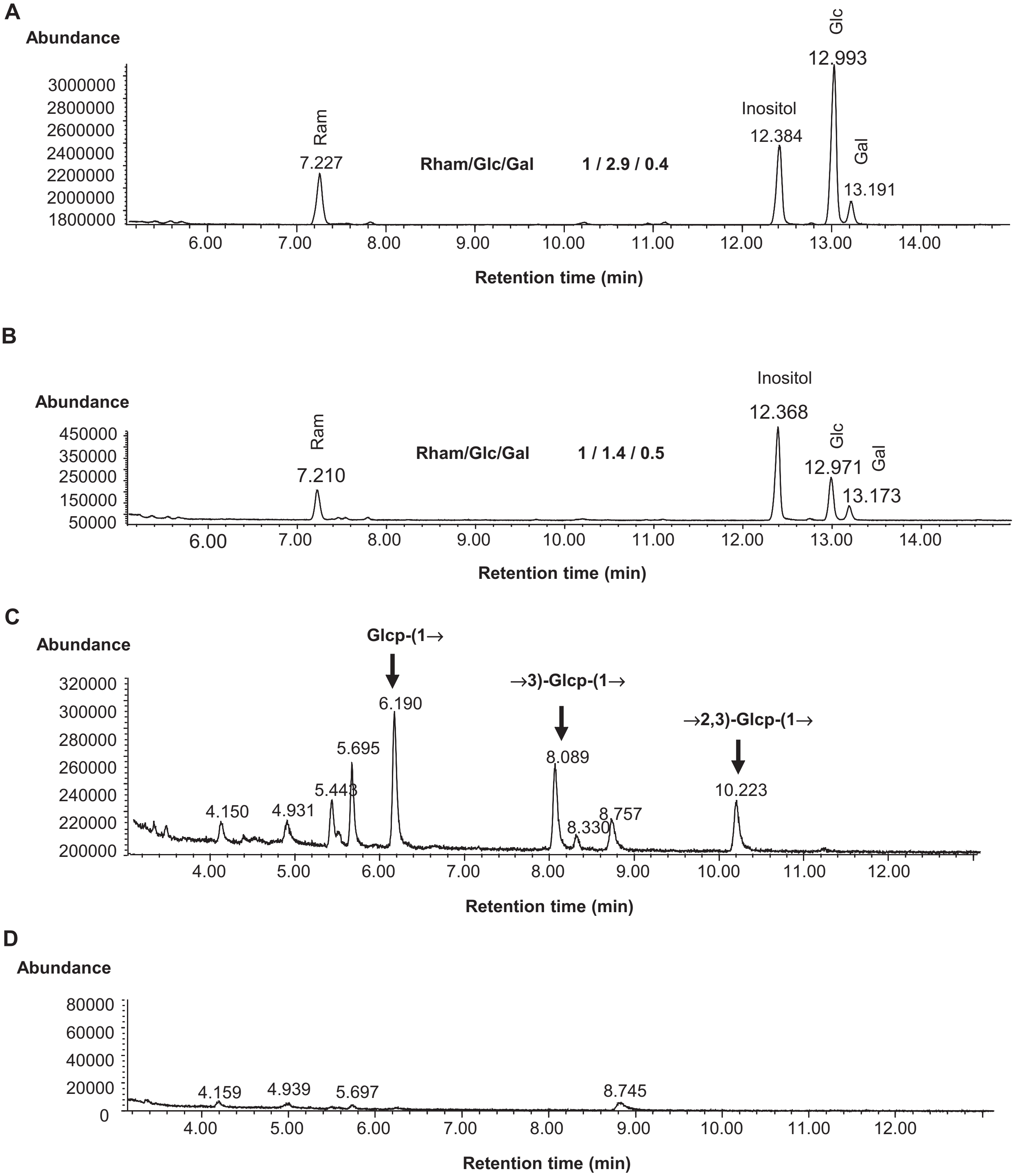

Fig. 5. Analysis by GLC of monosaccharides (A and B) and by GLC–MS (C and D) of linkage types between glucose units of the EPS synthesized *in vitro* by GTF glycosyltransferase. Analysis of reactions containing (A and C) or lacking (B and D) UDP-glucose.

3.3. *Quantification of EPS in growth media by using the ELISA assay or the phenol–sulphuric method*

The suitability of the immunological method for directly quantifying (1–3)-β-D-glucan in LAB growth media was tested as follows. The β-D-glucan purified from *L. lactis* NZ9000[pNGTF] was dissolved in either the DM (defined) or SDM (semi-defined) medium or in M17 and MRS complex media, and the EPS concentration was determined by the competition ELISA assay. In all cases the method was able to quantify EPS in the range of 30 to 500 ng ml^{-1} (Supplemental material S1). However, examination of the different slopes of the curves (see panels A; B, C and D of Supplemental material S1) in comparison with Fig. 2, indicated that for correct quantification of 2-substituted (1,3)-β-D-glucan in different buffers or growth media, the corresponding standard curve should be prepared using the corresponding solvent. Therefore, the method used to quantify β-D-glucan present in culture supernatants of LAB grown in the above mentioned media always used the appropriate standard curve. Thus, *L. lactis* NZ9000[pNGTF] and the isogenic β-D-glucan non-producing *L. lactis* NZ9000[pNZ8048] were grown in M17 medium as described previously (Werning et al., 2008). *P. parvulus* 2.6R and the isogenic β-D-glucan non-producing strain (2.6NR) as well as *O. oeni I4* and *Lb. diolivorans* G-77, and two other 2-substituted (1,3)-β-D-glucan producers, were grown in MRS medium to stationary phase as described above. The cultures were then centrifuged and, after removal of the supernatants, the EPS still bound to the cells were released by resuspension into one volume of PBS and the bacterial cells were removed by centrifugation. Then, the supernatants, after appropriate dilution in PBS, were directly used in the ELISA assay. The results revealed that the 2-substituted (1,3)-β-D-glucan secreted by the four producing-LAB could be detected in the media used, whereas only background levels were observed in the supernatants of the non-producing strains (Fig. 4).

To compare the inhibition enzyme immunoassay and the phenol–sulphuric method, culture supernatants of *L. lactis* NZ9000[pNGTF] grown in DM medium and *P. parvulus* 2.6R, *Lb. diolivorans* G-77 and *O. oeni* I4 grown in SDM medium were analyzed using both methods. Only these media were tested for detection of the 2-substituted (1,3)-β-D-glucan, because the presence of other polysaccharides in M17 and MRS media impairs the usage of the phenol–sulphuric method. The concentration of 2-substituted (1,3)-β-D-glucan was determined directly in the supernatants by the ELISA test or in their ethanol precipitates by the ELISA and phenol–sulphuric methods (Table 1). Determination of the EPS concentration in the precipitates showed that for all the strains tested the ELISA assay revealed a higher level of production (from 61% for *Lb. diolivorans* G-77 to 36% for *L. lactis* NZ9000[pNGTF]). In addition, comparison of EPS detection by the ELISA method prior to, or after, precipitation demonstrated that indeed precipitation results in a moderate loss of 2-substituted (1,3)-β-D-glucan in a range from 38% to 20% for *P. parvulus* 2.6R and *O. oeni* I4 biopolymers. These differences were probably due to low molecular weight EPS that was not precipitated by ethanol. Therefore, these results indicate that the ELISA method allows a more reliable determination of the biopolymer after its synthesis by bacteria.

3.4. *Detection of 2-substituted (1,3)-β-D-glucan produced in an enzymatic reaction*

Membrane vesicles containing the GTF glycosyltransferase, were prepared from *L. lactis* NZ9000[pNGTF] and the enzymatic activity was analyzed *in vitro* using UDP-glucose as substrate. The reaction product was detected and quantified by the ELISA method. Kinetic analysis of the results (Supplemental material S2) indicated that the GTF glycosyltransferase has an affinity for UDP-glucose of $K_m = 123 \pm 3\ \mu\text{M}$ and a $V_{\max} = 18 \pm 0.8$ ng of EPS/mg of total protein/min. This K_m is almost identical to that reported for the cellulose synthase BscA (125 μM) (Ross, Mayer, & Benziman, 1991) which, like GTF, belongs to the GT-2 family of glycosyltransferases.

To confirm the nature of the enzymatic reaction product, reactions performed with membrane vesicles and containing UDP-glucose (test) or lacking the substrate (control) were analyzed. In the presence or absence of the substrate, the GLC analysis detected rhamnose, glucose and galactose, in the ratios 1:2.9:0.4 and 1:1.4:0.5 for the test (Fig. 5A) and control (Fig. 5B) reactions, respectively. Thus, the *L. lactis* membrane vesicle preparations contained three monosaccharides, whose origin could be due to polysaccharides bound to them and as expected only glucose was highest in the test reaction. The GLC–MS analysis (Fig. 5C and D) detected, in the test reaction only (Fig. 5C), the three peaks that are characteristic of 2-substitute D-(1,3)-β-glucan, corresponding to terminal glucopyranose, 3-*O*-substituted glucopyranose, and 2-di-*O*-substituted glucopyranose. Thus, the product synthesized *in vitro* by the GTF glycosyltransferase was indeed 2-substituted (1,3)-β-D-glucan, confirming the ELISA detection of the product.

4. Discussion

ELISA tests have been developed previously for the detection of both linear and 6-substituted (1,3)-β-D-glucans (Douwes, Doekes, Montijn, Heederik, & Brunekreef, 1996; Sander, Fleischer, Borowitzki, Brüning, & Raulf-Heimsoth, 2008) as well as for the specific detection of 6-substituted (1,3)-β-D-glucans (Milton, Udeni Alwis, Fisette, & Muilemberg, 2001; Rao et al., 2007; Sander et al., 2008). However, as far as we know, this is the first instance of an ELISA test developed for the detection of 2-substituted (1,3)-β-D-glucan. In the inhibition enzyme immunoassay developed in this work the commercially available antibody (anti-*S. pneumoniae* serotype 37) has been used. Our results show that this primary antibody specifically detects 2-substituted (1,3)-β-D-glucan, has very low binding affinity for 6-substituted (1,3)-β-D-glucan and does not bind linear (1,3)-β-D-glucan even at a concentration as high as 500 $\mu\text{g ml}^{-1}$. The ELISA method allows detection of 30 ng ml^{-1} of the biopolymer in growth medium, a level of sensitivity similar to that observed for other β-D-glucan, such us (1,6)-β-D-glucan (Vink et al., 2004) or (1,3)-β-D-glucan (Douwes et al., 1996), when polyclonal antibodies were used for their detection. This method is less sensitive than that obtained for the detection of other polysaccharides with monoclonal antibodies (in the range of pg ml^{-1}) or than the amoebocyte lysate assays for which commercial kits, such as Glucatell® (Associates of Cape Cod, USA) or Fungitic-G (Seikagaku, Japan) are available. However, the first mentioned type of assay cannot be applied to the detection of 2-substituted (1,3)-β-D-glucan and the second type does not discriminate between different types of (1,3)-β-D-glucans. In addition, our results show that the sensitivity of our ELISA test permits the detection of novel 2-substituted (1,3)-β-D-glucan-producing bacteria by direct analysis of culture supernatants even after growth in complex media. Moreover, the method can be used for the biochemical characterization of the glycosyltransferases involved in their synthesis. These findings will facilitate the identification of new probiotic strains and the optimization of the production of this EPS.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at http://dx.doi.org/10.1016/j.carbpol.2014.06.072.

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Draft genome sequence of *Pediococcus parvulus* 2.6, a probiotic β-glucan producer strain (2016) *Genome Announcements* 4, e01381-16

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CHAPTER 2

Resumen

Pediococcus parvulus 2.6, una bacteria ácido láctica (BAL) aislada de sidra del País Vasco, considerada como prototipo de bacteria productora de un exopolisacárido tipo (1,3)-β-D-glucano ramificado en posición *O*-2 (β-glucano). El β-glucano es sintetizado por la GTF glicosiltransferasa utilizando como sustrato UDP-glucosa. Tanto la cepa 2.6 como su β-glucano han sido ampliamente estudiados y caracterizados. Se ha analizado el metabolismo de la bacteria y los factores que influyen en la síntesis de su EPS (Velasco et al., 2007), además de analizar las propiedades físico-químicas del EPS mediante resonancia magnética nuclear (Velasco et al., 2009). También, se ha estimado su masa molecular en más de 10^6 Da, y ensayos de reología mostraron que este β-glucano tenía potencial como bioespesante. Este β-glucano es producido solamente por un número reducido de BAL, cuyo nicho ecológico son las bebidas alcohólicas como el vino o la sidra. Así, su estructura y enlaces difieren de otros β-glucanos producidos por plantas, hongos o algas.

Por otra parte, se han caracterizado ciertas propiedades probióticas de *P. parvulus* 2.6, como su resistencia a un estrés gastrointestinal o la capacidad de adhesión *in vitro* a células humanas Caco-2 (Fernández de Palencia et al., 2009). Se ha mostrado que su EPS favorece la adhesión a las células epiteliales, mejora el crecimiento y viabilidad de algunos microorganismos probióticos (Russo et al., 2012) y favorece la proporción antiinflamatoria IL10/TNFα en un modelo *in vitro* de macrófagos humanos (Notararigo et al., 2014).

Teniendo en cuenta estos antecedentes, la secuenciación del genoma de *P. parvulus* 2.6 supone un paso más en la consecución de una visión global de su potencial metabólico, así como valorar la presencia de determinantes incompatibles con su potencial utilización como bacteria probiótica. Además, ha supuesto la caracterización del primer genoma de una estirpe de *P. parvulus*.

La secuenciación del genoma se llevó a cabo a partir de una muestra de 2 µg de ADN genómico. Una librería del genoma completo se obtuvo mediante la técnica TruSeq ADN y utilizando el sistema Genome AnalyzerIIx de Illumina. El análisis de la librería permitió ensamblar el genoma en 115 cóntigos, el menor de 206 pb y el mayor de 171 kpb. También permitió estimar el tamaño del genoma en 2.236 kpb. Se anotaron un total de 2.241 genes de los cuales 2.069 codifican proteínas, 3 ARNs ribosomales, 60 ARNs transferentes, 4 ARNs no codificantes y 105 pseudogenes. El genoma completo de *P. parvulus* 2.6 fue depositado en el GenBank bajo el número de acceso LXND00000000.

Mi aportación a este trabajo

Para lograr la consecución de este trabajo científico, mi aportación fue la siguiente. Contribuí a la extracción del ADN genómico bacteriano que fue objeto de la secuenciación y posteriormente contribuí al análisis de la secuencia obtenida detectando el operón de utilización y transporte de sorbitol, que fue estudiado en el Capítulo 4 de esta tesis. Además, realicé el borrador del manuscrito.

AMERICAN SOCIETY FOR MICROBIOLOGY | genomeAnnouncements

Draft Genome Sequence of *Pediococcus parvulus* 2.6, a Probiotic β-Glucan Producer Strain

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We report here the draft genome sequence of the probiotic *Pediococcus parvulus* 2.6, a lactic acid bacterial strain isolated from ropy cider. The bacterium produces a prebiotic and immunomodulatory exopolysaccharide, and this is the first strain of the *P. parvulus* species whose genome has been characterized.

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We report here the draft genome sequence of *Pediococcus parvulus* 2.6 (formerly *Pediococcus damnosus*), a lactic acid bacterium isolated from ropy cider (1). This is the first strain of *P. parvulus* whose genome has been characterized. The 2.6 strain produces the immunomodulatory exopolysaccharide 2-substituted (1,3)-β-D-glucan (2), which is synthesized by a limited number of bacteria and confers probiotic properties to the producing strains. This polysaccharide differs from the β-glucans produced by plants and other microorganisms. The synthesis of 2-substituted (1,3)-β-D-glucan by *P. parvulus* 2.6 is controlled by a single heterotransmembrane glucosyltransferase (GTF), which polymerizes glucosyl residues from UDP glucose (3–5). Some of the conditions that influence the homopolysaccharide (HoPS) synthesis by *P. parvulus* 2.6, as well as the enzymatic activities involved in sugar metabolism in this strain, have been examined (6, 7). The physicochemical properties of the HoPS have also been determined, and nuclear magnetic resonance (NMR) analysis showed that the same HoPS was synthesized, irrespective of the sugar source used for growth (8). The molecular masses of these β-glucans are high (>10^6 Da), and their rheological properties showed that they have potential utility as biothickeners (8). An oat-based product fermented by *P. parvulus* 2.6 showed improved rheology (9), thereby decreasing the need for added stabilizers and texturizers.

The potential of *P. parvulus* 2.6 as a probiotic strain has also been examined; it resists gastrointestinal stress, adheres to Caco-2 cells, and its HoPS reduces the production of inflammatory cytokines by polarized macrophages (10). We have also shown that *P. parvulus* HoPS improves the growth and viability of probiotic microorganisms, as well as their adhesion to human enterocytes (11). The 2-substituted (1,3)-β-D-glucan increases *in vitro* the ratio of interleukin 10 (IL10) (anti-inflammatory) to tumor necrosis factor alpha (TNFα) (inflammatory) in human macrophages and decreases the levels of the proinflammatory IL8 in human intestine organ cultures (reference 12 and Notararigo S., Antolin M., Guarner F., López P., unpublished data).

An oat-based product fermented by *P. parvulus* 2.6 had a bifidogenic effect and decreased serum cholesterol levels in humans (13). Possibly, the 2-substituted (1,3)-β-D-glucan has a synergistic effect with the hypocholesterolemic action of oat β-glucans. Finally, *P. parvulus* 2.6 displays antibacterial activity against several bacterial species (14), including natural contaminants in oats, a property that reduces the need for chemical preservatives and improves the functionality of the final product.

Two micrograms of genomic DNA was subjected to library preparation using the TruSeq DNA sample perp kit FC-121-1001, according to the manufacturer's instructions. Whole-genome sequencing used the Illumina GAIIx at the Genomics Research Centre (Fiorenzuolad'Arda, Italy). A total of 26,018,224 paired-end reads (2 × 110-bp length) were assembled into 115 contigs. The genome was calculated to be 2,236,754 long. The size of the shortest contig was 206 bp, while the length of the longest contig was 171,226 bp. The genome sequence was annotated by the NCBI Prokaryotic Genomes Annotation Pipeline. A total of 2,241 genes were predicted to encode 2,069 proteins, three rRNAs, 60 tRNAs, and four noncoding RNAs (ncRNAs), and 105 are pseudogenes.

Accession number(s). The complete genome of *P. parvulus* 2.6 has been deposited at DDBJ/EMBL/GenBank under accession number LXND00000000.

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Construction and validation of a mCherry protein vector for promoter analysis in *Lactobacillus acidophilus*. (2015) *Journal of Industrial Microbiology and Biotechnology* 42: 247-253

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CHAPTER 3

Resumen

El género *Lactobacillus* es uno de los más abundantes dentro del grupo de las bacterias del ácido láctico (BAL), y también de los más importantes debido a su interés biotecnológico, así como por sus características probióticas que promueven efectos beneficiosos para la salud. Así, para realizar su evaluación es útil el desarrollo de nuevas herramientas genéticas que permitan estudiar la expresión de genes que codifiquen enzimas y otras proteínas de interés. Entre dichas herramientas, cabe destacar los vectores plasmídicos para evaluar promotores transcripcionales, que contienen genes codificantes de proteínas fluorescentes. Además, cuando los vectores contienen fusiones transcripcionales, son responsables de la expresión de dichas proteínas y pueden ser utilizados, una vez transferidos, para la detección y localización de bacterias en procesos interactivos como la formación de biopelículas o la colonización del tracto gastrointestinal. En un trabajo previo de nuestro grupo, se diseñó un gen *mrfp* sintético, que codifica la proteína mCherry, una variante monomérica de la proteína fluorescente roja de *Dicosoma* sp. Los codones del gen *mrfp* fueron optimizaron para su expresión en bacterias Gram-positivas, y la proteína mCherry codificada por él, a partir de plásmidos recombinantes, fue validada como marcador en *Lactococcus lactis* y *Enterococcus faecalis* (Garcia-Cayuela et al., 2012).

Sin embargo, los plásmidos construidos en el trabajo antedicho, que contenían dos replicones distintos, no fueron funcionales ni en lactobacilos ni en neumococos. Por ese motivo, en este trabajo, se construyó el plásmido denominado pRCR, que permite la evaluación de promotores en cepas pertenecientes a especies del género *Lactobacillus* y en *Streptococcus pneumoniae*. Este plásmido, que replica mediante el mecanismo de circulo rodante, contiene el replicón pSH71 de *L. lactis* y presenta un amplio espectro de hospedador. El plásmido pRCR porta, además del gen *cat* que codifica la cloranfenicol acetil transferasa, el gen *mrfp* precedido por un sitio para clonación múltiple. Fue generado *in vitro* y establecido en *S. pneumoniae* 708 mediante electrotransformación. Los transformantes fueron seleccionados por resistencia a cloranfenicol, y posteriormente fue transferido a *Escherichia coli* DH5α. También, los resultados presentados en el Capítulo 4 de esta tesis han demostrado la validez del vector para estudios de expresión génica en *Lactobacillus plantarum* y *Lactobacillus casei*.

En este trabajo, para validar la mCherry como proteína marcadora de la expresión génica en los lactobacilos, se clonó la posible región promotora P_{lbaB} de *Lactobacillus acidophilus* en el vector pRCR generando el plásmido pRCR11. Este plásmido fue establecido en *E. coli* DH5α, y transferido posteriormente a *L. acidophilus* CECT903. El gen *lbaB* de *L. acidophilus* codifica la bacteriocina lactacina B y se ha demostrado previamente que su expresión es inducible por el co-cultivo con otras bacterias vivas, como *Streptococcus thermophilus* (Tabasco et al., 2009). Así, en este trabajo se evaluó la expresión de la proteína mCherry tanto en cultivo simple, como en co-cultivo con *S. thermophilus* STY-31, mediante medidas espectrofotométricas de la fluorescencia emitida por la bacteria portadora. El crecimiento de la cepa CECT903 fue similar en ambas condiciones. Sin embargo, solamente se obtuvo señal fluorescente en la condición de co-cultivo, donde el incremento de la señal apareció ligado al crecimiento y alcanzó su valor máximo durante la fase estacionaria. Los resultados obtenidos mostraron que la regulación de la expresión de la lactacina B se inicia desde el comienzo de la fase exponencial. Además, revelaron la existencia de un promotor funcional en el fragmento de ADN clonado en pRCR11. En este trabajo, también nos propusimos caracterizar este promotor, analizando el inicio de la transcripción mediante la técnica de extensión del cebador. Los resultados revelaron dos fragmentos extendidos de 185 (el más abundante en co-cultivo) y 187 nucleótidos, correspondientes respectivamente a dos posibles sitios de inicio de la transcripción, una citosina y una adenina. Con las muestras en co-cultivo con *S. thermophilus*, se detectaron en la posición 185 niveles del fragmento extendido 5 veces superiores a los obtenidos con los monocultivos de *L. acidophilus*. Aguas arriba del sitio de inicio de la transcripción se localizaron las cajas -35 (TTGtAa) y -10 (aATAAT) características de promotores bacterianos. Además, los dos sitios de inicio de la transcripción están incluidos en uno de los brazos de una repetición invertida situada aguas abajo del promotor, característica del sitio de unión de un regulador transcripcional y por su localización, característica de un represor. La expresión del operón de la lactacina B está controlada por el regulador de respuesta RR_1798 (RR) de un sistema de tres componentes (Altermann et al., 2005; Tabasco et al., 2009). Así, en condiciones de monocultivo, el RR actuaría como un represor impidiendo la unión de la ARN polimerasa al promotor por competición, produciéndose bajos niveles de transcripción. Por el contrario, la presencia de otra bacteria sería detectada por la histidina kinasa HK_1799 del sistema, que modificaría al RR fosforilándolo, inhibiendo la represión transcripcional y provocando un incremento en la expresión de *lbaB*.

Mi aportación a este trabajo

Para lograr la consecución de este trabajo científico, mi aportación fue la siguiente. Contribuí al estudio de la expresión génica a partir del promotor P_{lbaB}, a la realización de las inducciones en co-cultivo y a la extracción de las muestras de ARN para los estudios de extensión del cebador.

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BIOTECHNOLOGY METHODS

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Construction and validation of a mCherry protein vector for promoter analysis in *Lactobacillus acidophilus*

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Abstract Lactobacilli are widespread in natural environments and are increasingly being investigated as potential health modulators. In this study, we have adapted the broad-host-range vector pNZ8048 to express the mCherry protein (pRCR) to expand the usage of the mCherry protein for analysis of gene expression in *Lactobacillus*. This vector is also able to replicate in *Streptococcus pneumoniae* and *Escherichia coli*. The usage of pRCR as a promoter probe was validated in *Lactobacillus acidophilus* by characterizing the regulation of lactacin B expression. The results show that the regulation is exerted at the transcriptional level, with *lbaB* gene expression being specifically induced by co-culture of the *L. acidophilus* bacteriocin producer and the *S. thermophilus* STY-31 inducer bacterium.

Keywords *Lactobacillus* · Broad-host-range vector · pRCR · Bacteriocin · Promoter regulation

Introduction

Lactobacilli belong to the lactic acid bacteria (LAB) group, and traditionally they have been used for fermented food production. They are widespread in natural environments including niches such as the human and animal gastrointestinal tracts, where they can play important roles as health modulators [2, 23]. Thus, they are increasingly used as functional food ingredients (probiotics), and as vectors for live oral vaccines and drugs [3, 24, 25]. Several *Lactobacillus* species have been identified to produce bacteriocins, some of them have a dual role, acting as inhibitors at high concentrations and participating in interspecies communication or bacterial crosstalk [8].

Due to the increased interest in lactobacilli health effects, it is relevant to develop genetic tools that can detect regulation in living cells of gene expression such as the production of proteins, including enzymes, from these bacteria [9, 15]. One strategy is the construction of fluorescent proteins to study the effect of lactobacilli in the immune system and their localization within the gastrointestinal tract [2, 6]. Fluorescent proteins could also facilitate the monitoring of these bacteria in biofilm formation and interaction with the host. In addition, fluorescent proteins are used as reporters for transcriptional gene expression and regulation. In our previous work, we designed a *mrfp* gene codon, optimized for expression in Gram-positive bacteria, from the monomeric variant of the 'mCherry' red fluorescent protein (RFP) from *Dicosoma* sp. [11]. Its use as a reporter in *Lactococcus lactis* and *Enterococcus faecalis* was validated by the construction and testing of shuttle vectors based on the pAK80 plasmid [11]. Additional applications of the mCherry-derived vectors in these LAB species have been recently reported [4, 5].

M.L. Mohedano and T. García-Cayuela contributed equally to the work.

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In this study, we have constructed a vector based on the pSH71 replicon [7, 12], to expand the usage of mCherry for analysis of gene expression in *Lactobacillus* and its usage as a promoter probe validated in *L. acidophilus*.

Materials and methods

Bacterial strains and culture conditions

Lactobacillus acidophilus CECT 903 from the Colección Española de Cultivos Tipo (Paterna, Spain) and *L. acidophilus* La5 (Chr. Hansen, Hørsholm, Denmark) strains were grown in MRS broth (Pronadisa, Madrid, Spain) supplemented with 0.05 % L-cysteine hydrochloride (Calbiochem, Merck KGaA, Darmstadt, Germany) and 0.2 % Tween 80 (Oxoid, Hampshire, UK) (MRSCT) at 37 °C. *Streptococcus thermophilus* STY-31 (Chr. Hansen) was grown in ESTY broth (Pronadisa) supplemented with 0.5 % glucose at 37 °C. *Escherichia coli* DH-5α [21] was grown in Luria–Bertani broth at 37 °C with vigorous shaking. *Streptococcus pneumoniae* 708 [18] was grown in AGCH medium [17] supplemented with 0.25 % yeast extract and 0.8 % sucrose at 37 °C without shaking. When necessary, chloramphenicol (Sigma-Aldrich, St. Louis, MO, USA) was added to the culture medium at a final concentration of 10 μg mL^{-1} for *E. coli* and 5 μg mL^{-1} for *S. pneumoniae* and *L. acidophilus*. Plate media were prepared by adding agar (Pronadisa) to liquid broth at a final concentration of 1.5 %.

General DNA manipulation and transformation

The promoter-probe vector and the expression plasmid constructed in this work were based on the pSH71 replicon [7, 12]. Plasmid pRCR was constructed as follows (Fig. 1). Plasmid pNZ8048 [16] was digested with *Bgl*II and *Sac*I to remove the nisin promoter. The resultant 3,168 bp DNA fragment, containing the rolling-circle replicon of the pSH71 plasmid and the chloramphenicol resistance *cat* gene, was purified from a 0.8 % agarose gel using the QIAquick Gel Extraction Kit (Qiagen Iberia, Madrid, Spain). The *mrfp* gene encoding the mCherry protein [11] was amplified from plasmid pTVCherry (National Collections of Industrial and Marine Bacteria, Aberdeen, UK) by using the specific primers mCherryF (5′-GGAAGATCTTCCCGAATTCCCCGGGGATCCTCTAGAGGGATACGCACG AGTTTCAACT-3′) and mCherryR (5′-CGCGAGCTCATTTATATAATTCGTCCATGCCACCTGT-3′) to obtain a 801 pb amplicon containing the *mrfp* gene preceded by the multicloning site *Bgl*II, *Eco*RI *Sma*I, *Xma*I *Bam*HI, *Xba*I. The PCR product was then digested with *Bgl*II and *Sac*I restriction enzymes and ligated, with T4 DNA ligase (Thermo Fisher Scientific, Waltham, USA), to the 3,168 bp fragment from pNZ8048. The resulting plasmid, named pRCR (3,960 bp), was established in *S. pneumoniae* 708 by transformation as previously described [19]. Transformants were selected for chloramphenicol resistance, and the correct nucleotide sequence of the insert, containing a multicloning site and the *mrfp* gene in pRCR, was confirmed by DNA sequencing at Secugen S.L. (Centro de Investigaciones Biológicas, Madrid, Spain).

The promoter (P_{lbaB}) of the *L. acidophilus* La5 lactacin B gene [22] was cloned upstream of *mrfp* in pRCR, generating the transcriptional fusion P_{lbaB}-*mrfp* in pRCR11. To this end, an amplicon was generated using the chromosomal DNA of *L. acidophilus* La5 as template and the primers LBA1797F (5′-AGGAGATCTGCGTACAAAGATGTGGTTAA-3′) and LBA1797R (5′-AGGTCTAGATGAGATTTTTATCTCATTTCAAC-3′) to obtain a 221 pb fragment containing the P_{lbaB} sequence. After digestion with *Bgl*II and *Xba*I, the amplicon was introduced into the multicloning site of pRCR, after digestion with the same restriction enzymes. The resulting plasmid pRCR11 (4,166 bp) was established in *E. coli* DH5α by transformation as described previously by Hanahan [13]. The presence of the transcriptional fusion in pRCR11 was confirmed by DNA sequencing with primers LBA1797F and LBA1797R.

Plasmid pRCR11 was transferred to *L. acidophilus* CECT903 cells by electrotransformation as follows. The CECT903 strain was grown under aerobic conditions at 37 °C, without shaking, during 15 h in MRSCT broth supplemented with 1 % glycine. Subsequently, 4 mL of the culture were used to inoculate 200 mL of the same fresh medium, and grown until it reached an OD_{600} of 0.3–0.4. Cells were collected by centrifugation at 0 °C and 8,600×*g* for 10 min and washed four times with ice-cold electroporation buffer (HEPES 0.1 mM, sucrose 0.5 M, pH 7.5). Finally, cells were resuspended in 1.6 mL of ice-cold electroporation buffer. 750 μL of cells and 0.5 μg of plasmid DNA were used for electrotransformation. The electroporation conditions were 25 μF, 200 Ω and 2.5 kV in a 0.4-cm cuvette, using a Gene Pulser and a Pulse Controller apparatus (Bio-Rad, Richmond, CA, USA). After electroporation, cells were resuspended in 8 mL of MRSCT broth supplemented with 10 mM $CaCl_2$ and 0.5 M sucrose, incubated aerobically at 37 °C for 3 h, without agitation, and then plated onto MRSCT medium supplemented with 1 % agar and chloramphenicol.

Induction assay and determination of fluorescence

Induction of expression of mCherry from the P_{lbaB} promoter in *L. acidophilus* CECT903[pRCR11] cells was assayed by co-culturing *L. acidophilus* CECT903[pRCR11] with the inducer *Streptococcus thermophilus* STY-31 strain [22].

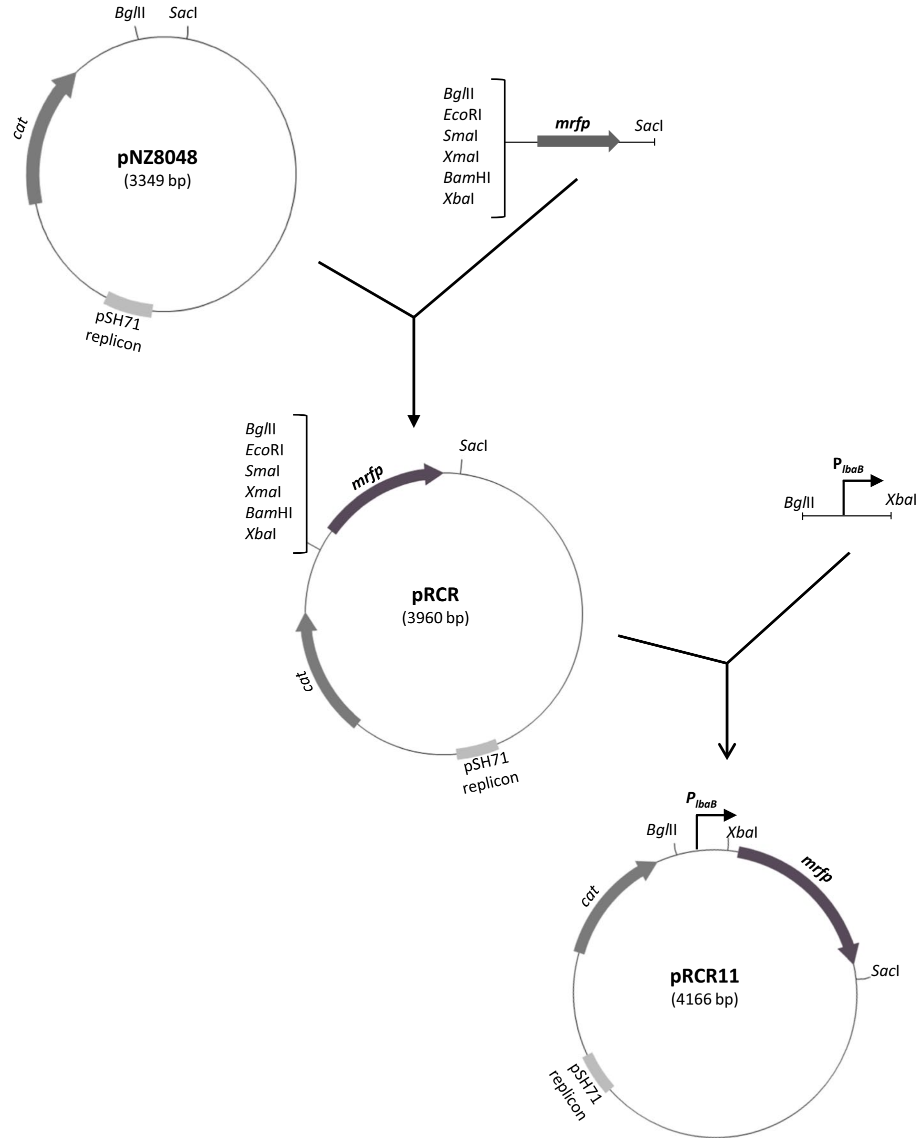

Fig. 1 Schematic diagram showing the construction of pRCR and pRCR11. For details, see "Materials and methods". Relevant restriction sites are shown. Specific genes are: *mrfp* and *cat* that encode mCherry and the protein responsible for the resistance to chloramphenicol, respectively. P_{lbaB}, promoter of the lactacin B structural gene of *Lactobacillus acidophilus* La5

Co-cultures were carried out in MRSCT medium inoculated with 2 % each of *L. acidophilus* CECT903 [pRCR11] and *S. thermophilus* STY-31 overnight cultures.

The levels of fluorescence of the mCherry encoded by pRCR11 and bacterial growth were tested simultaneously with the Varioskan Flash system (Thermo Fisher Scientific, Waltham, MA, USA), which provides quantitative data of cell density via measuring OD at 600 nm and in vivo mCherry expression at an excitation wavelength of 587 nm and an emission wavelength of 612 nm. Background fluorescence of the control strain (*L. acidophilus* CECT903 [pRCR11] without *S. thermophilus* STY-31 grown under the same conditions) was used to normalize the fluorescence signals during cultivation. All measurements were performed in sterile 96-well optical bottomed microplates (Nunc, Rochester, NY, USA) with a final assay volume of 300 μL per well by using the microtiter plate assay system Varioskan Flash. The microplates were incubated for 24 h at 37 °C. Measurements were made at 1 h intervals.

Preparation of nucleic acids

For cloning and sequencing experiments, plasmidic DNA was purified from *E. coli* DH-5α by usage of QIAprep Spin Miniprep and Midiprep kits (Qiagen, Hilden, Germany) or from *S. pneumoniae* 708 as previously described [21]. For primer extension analysis, a culture of *L. acidophilus* CECT903[pRCR11] and the co-culture of *L. acidophilus* CECT903 [pRCR11] and *S. thermophilus* STY-31 were grown to an OD$_{600}$ of 1.2 and then used for analysis of *mrfp* mRNA. Total RNA was isolated with a Ribolyser and Recovery kit from Hybaid (Middlesex, UK) as specified by the supplier. The RNAs were checked for the integrity and yield of the rRNAs by Qubit™ fluorometer (Invitrogen, Madrid, Spain) and by Gel Doc 1000 (Bio-Rad). The patterns of rRNAs were similar in all preparations.

Primer extension analysis

Primer extension analysis was performed by a modification of the method described by Fekete et al. [10]. The start site of *lbaB* mRNA was detected using the LBABP primer (5′-TGAGTTGAAACTCGGTGCGTATCCTCT-3′) labeled with 6-FAM at its 5′-end (Sigma-Aldrich). Two hundred picomoles of primer were annealed to 40 µg of total RNA. Primer extension reactions were performed by incubation of the annealing mixture with 20 nmol each of dNTP (dATP, dGTP, dCTP and dTTP), 200 U of Maxima Reverse Transcriptase (Thermo Fisher Scientific, Madrid, Spain) in 1× reverse transcriptase buffer (Thermo Fisher) in a final volume of 50 µL at 50 °C for 60 min. Then, the reactions were supplemented with 50 µL of TE (10 mM Tris HCl pH 8.0, 1 mM EDTA) and purified by treatment with phenol (vol:vol) for 5 min at room temperature and ethanol precipitation with three volumes of 100 % ethanol in the presence of 0.3 mM Na acetate. After overnight storage at −20 °C, samples were sedimented by centrifugation at 12,000×*g* for 30 min at −10 °C and resuspended in TE (12 µL).

Detection and quantification of the reaction products were carried out in a 8 % polyacrylamide gel containing 7 M urea. Bands labeled with 6-FAM were detected and directly quantified with a FujiFilm Fluorescent Image Analyzer FLA-3000 (Fujifilm, Düsseldorf, Germany).

For determination of the length of the extended products, the primer extension reactions were further purified using Agencourt Clean Seq (Beckam Coulter, Alcobendas, Madrid, Spain) and kept frozen at −20 °C until use. Samples were separated on an Abi 3730 DNA Analyzer (Applied Biosystems, Tres Cantos, Madrid, Spain) capillary electrophoresis instrument using techniques and parameters recommended by the manufacturer. A DNA sequence of pRCR11 determined by the dideoxynucleotide method with unlabeled LBABP primer was included in the same capillary in each run to determine fragment length. The Peak Scanner version v1.0 (Applied Biosystems) was used to screen the data and identify major peaks.

Results and discussion

Plasmids containing the mCherry coding gene

Following the construction and analysis of the pTL family of plasmids designed for using mCherry as a reporter in LAB [11], we were unable to transfer any of these plasmids neither to *L. casei* and *L. acidophilus* strains nor to *S. pneumoniae* strains. These pTL plasmids were derived from pAK80, which carries, in addition to the erythromycin resistance marker, two origins of replication, one from the lactococcal plasmid pCT1138 and the other from the *E. coli* p15A plasmid, and replicates in Gram-positive bacteria by the theta mode mechanism [14]. As an alternative, and with the aim of developing new tools for gene expression analysis in lactobacilli, the use of the replicon of the *L. lactis* pSH71 plasmid [7, 12] was investigated. This plasmid uses the rolling-circle-type mechanism for replication, and is characterized by a broad host-range, which includes Gram-positive bacteria and *E. coli*. Therefore, a promoter-probe vector (pRCR) and an expression plasmid (pRCR11) carrying the synthetic *mrfp* gene optimized for LAB [11] and the chloramphenicol resistance marker were constructed (Fig. 1). pRCR was generated by changing a DNA fragment of pNZ4048 containing the *nisA* promoter, located between the restriction sites *Bgl*II and *Sac*I, by a DNA fragment containing the *mrfp* gene preceded by a polylinker to facilitate further cloning of transcriptional promoters upstream of the mCherry coding gene. The pRCR plasmid was established in *S. pneumoniae* 708 by transformation and selection for chloramphenicol resistance. In addition, as predicted, the plasmid was also able to replicate in *L. sakei* MN1, *L. plantarum* WCFS1 and *E. coli* DH5α (personal communications by M. Nacher, A. Pérez-Ramos and M.L. Mohedano, respectively). Consequently, the resulting vector had kept its broad-host-range attribute and had the potential to be used in various LAB species.

To evaluate the functional expression of mCherry in lactobacilli, the region located upstream of the *lbaB* bacteriocin structural gene from *L. acidophilus* [22] and carrying the putative promoter P_{lbaB} was cloned upstream of *mrfp* in pRCR to generate pRCR11. The expression of lactacin B in *L. acidophilus* has been demonstrated to be inducible by the co-culture with live target bacteria [22]. The *mrfp* gene was used as reporter to monitor the P_{lbaB} activity during the induction of lactacin B production by the transformation of *L. acidophilus* CECT 903 with pRCR11. The induction of bacteriocin expression was assayed by co-culturing this strain with *S. thermophilus* STY-31, a previously identified inducer strain [22]. The functional expression of mCherry under the control of P_{lbaB} and the increase of biomass during cell growth were monitored (Fig. 2). The results revealed that the growth of *L. acidophilus* CECT 903[pRCR11] was very similar in both single and co-cultures. However, the mCherry activity was detected only in the presence of *S. thermophilus* STY-31. Moreover, under co-culture conditions the increase of mCherry fluorescence correlated with the growth pattern. These results indicated that up-regulation of lactacin B expression initiates during exponential growth as previously demonstrated [22]. The maximum fluorescence levels were detected at the

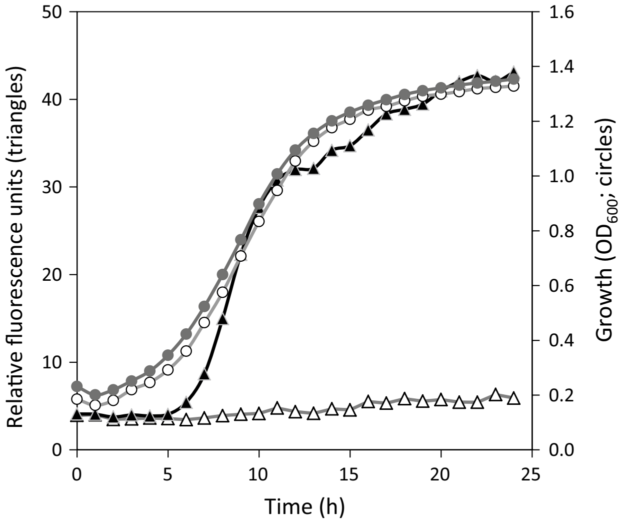

Fig. 2 Detection of induction of expression of mCherry encoded by pRCR11. Fluorescence (relative fluorescence units; *triangles*) and growth (OD$_{600}$; *circles*) of cultures of *L. acidophilus* CECT 903[pRCR11] (*open symbols*) and co-cultures of *L. acidophilus* CECT 903[pRCR11] and *Streptococcus thermophilus* STY-31 (*closed symbols*) grown in MRSCT are depicted. The growth of cultures was monitored at a wavelength of 600 nm. Fluorescence emission of mCherry was recorded at 612 nm after excitation at a wavelength of 587 nm

stationary phase (OD$_{600}$ = 1.3), consequently growth to this phase was used for further experiments.

Transcriptional analysis of the influence of co-culture with *S. thermophilus* on lactacin B expression in *L. acidophilus*

Our previous quantitative RT-PCR studies of *L. acidophilus* La5 *lbaB* gene expression had shown that in the presence of *S. thermophilus* STY-31 an increase of the *lbaB* transcript takes place [22]. Thus, total RNA was extracted from *L. acidophilus* CECT603[pRCR11] cells grown in the presence or absence of *S. thermophilus* STY-31 to stationary phase and samples were used for primer extension analysis performed with a 5-end 6-FAM labeled LBABP primer. Analysis of the same volume (6 μL) of both reactions in a polyacrylamide gel detected the extended products encoded by pRCR11 complementary to the *lbaB* transcript in *L. acidophilus* CECT603[pRCR11] grown in the presence or absence of *S. thermophilus* STY-31 (Fig. 3b). In the absence of the inducer two bands with similar intensity were observed, whereas in the co-cultures one more prominent and two minor longer extended products were observed. The fluorescence of the bands was quantified with a fluorescent image analyzer, and the results revealed a fivefold induction due to the presence of *S. thermophilus* STY-31 (Fig. 3b).

To determine the length of the extended products and the 5-end of the *lbaB* transcript, the primer extension reactions were also analyzed by capillary electrophoresis in conjunction with a DNA sequence of pRCR11 generated with unlabeled LBABP primer (Fig. 3a). Since we expected low transcript levels of P_{lbaB} in cells grown in mono-culture, we processed 250 nL for the capillary electrophoresis experiments compared to 40 nL derived from cells grown in co-culture. The pattern of the peaks observed (Fig. 3b) correlated with that obtained for the labeled bands in the polyacrylamide gel (Fig. 3a). The two bands detected in cultures of *L. acidophilus* CECT603[pRCR11] corresponded to extended products of 185 and 187 nt, the first being the major band present in the co-cultures (Fig. 3b). This result located the 5′-end of the *lbaB* mRNA at a C and A (Fig. 3c), since 6-FAM labeled DNA extended products run as if they were, on an average, three nucleotides shorter than the dideoxy sequencing products [10]. Upstream of the start sites, a putative promoter was detected composed of a −35 (TTGtAa) and a −10 (aATAAT), these sequences being characteristic for the binding of the vegetative σ factor of the bacterial RNA polymerases with an anomalous (too long) spacing of 24 nt (Fig. 3c). Moreover, the two start sites for transcription are included in one of the arms of the inverted repeat characteristic for binding of transcriptional regulators. This location predicts that the binding of a protein to the inverted repeat will impair initiation of transcription catalyzed by the RNA polymerase. The expression of the lactacin B operon is regulated by the response regulator RR_1798 which is part of a three-component regulatory system composed of the inducing peptide IP_1800, the HK_1799 histidine kinase and the RR_1798 response regulator [1, 22]. Thus, it seems that under uninduced conditions competition between the RNA polymerase and RR_1798 for binding to the upstream region of *lbaB* gene will result in low levels of the transcript starting at the two nucleotides G and C. Then, in the presence of bacteria that compete for the environmental niche, HK_1799 would sense its presence and, by modification of the RR_1798, would impair the repression of transcription of *lbaB* and result in an increase of lactacin B levels. We have previously demonstrated that the production of lactacin B by *L. acidophilus* is controlled by an autoinduction mechanism involving a secreted peptide and by co-culture with live inducer cells [22]. These characteristics of induction of bacteriocin production through autoinduction and co-culture have been recently described to be widespread among bacteriocinogenic *L. plantarum* strains [20]. The use of mCherry as a promoter probe in pRCR11 has allowed us to locate the region where the *lbaB* transcriptional regulation is specifically induced by co-culture of the lactacin B producer with the inducing bacteria.

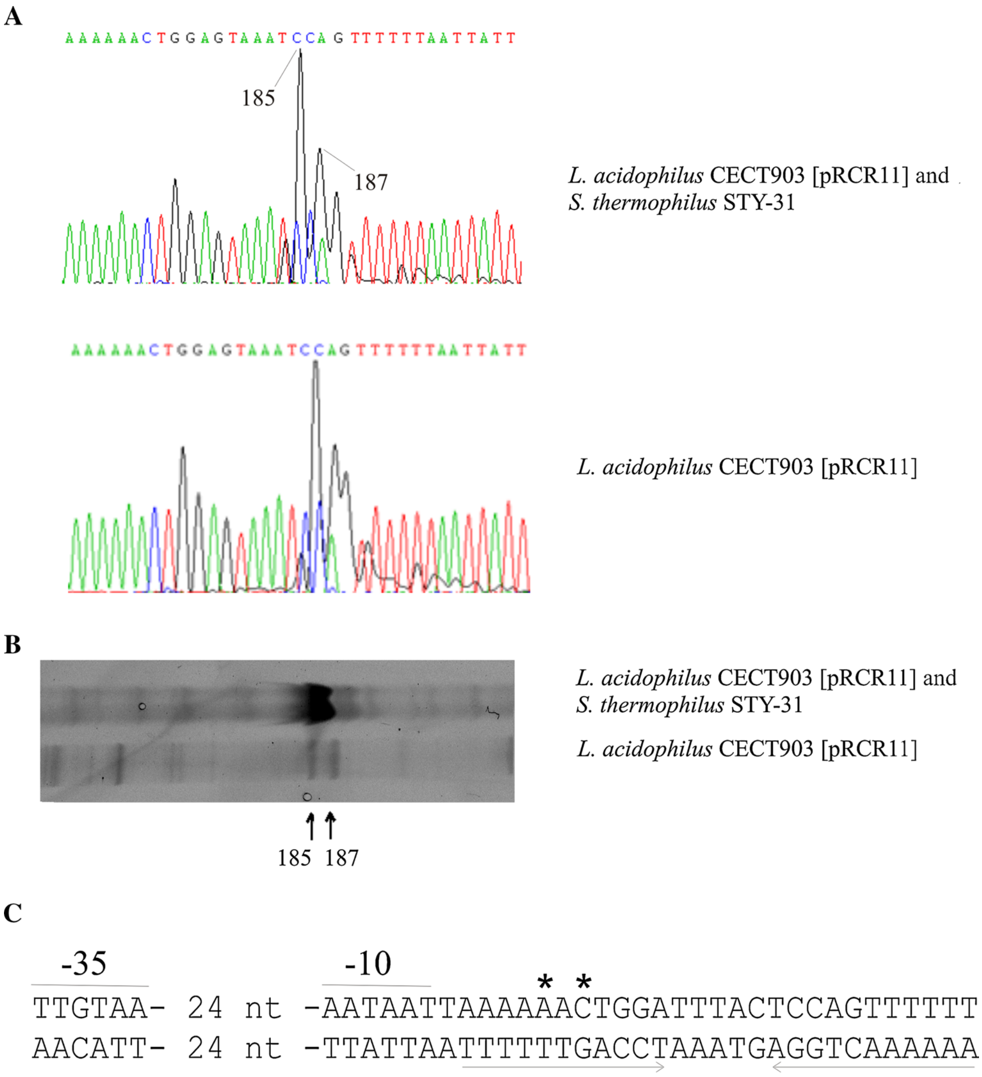

Fig. 3 Detection of the start site of the *lbaB* transcript by primer extension. Reactions containing total RNA isolated from cultures of *L. acidophilus* CECT 903[pRCR11] alone or in co-culture with *S. thermophilus* STY-31 were analyzed by capillary electrophoresis in conjunction with DNA sequence of pRCR11 (**a**) or by 8 % denaturating polyacrylamide gel electrophoresis (**b**). For primer extension and DNA sequence analysis, primers fluorescently labeled at the 5′-end with 6-FAM, or unlabeled (both having the same DNA sequence) were used, respectively. Extended products ran as if they were, on an average, three nucleotides shorter than the dideoxy sequencing products. The length of the extended products determined by the analysis depicted in **a** is indicated in the analysis showed in **b**. The DNA region surrounding the start site of the mRNA is also depicted (**c**). The start sites of the transcript detected in **a** are indicated by *stars*. The −35 and −10 regions of the P_{lbaB} promoter are shown. The inverted repeat, putative binding site of the RR_1798 response regulator, is indicated by *arrows*

In conclusion, the rolling-circle-type mechanism for replication of pRCR has broadened the host-range in LAB of the mCherry-based vectors pTLR. Indeed, the promoter-probe vector pRCR has demonstrated to be suitable for characterization of complex promoter induction mechanisms such as those related to bacteriocin production by *L. acidophilus*.

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Characterization of the sorbitol utilization cluster of the probiotic *Pediococcus parvulus* 2.6: genetic, functional and complementation studies in heterologous hosts. (2017) *Frontiers in Microbiology* 8, 2393

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CHAPTER 4

Resumen

Las bacterias del ácido láctico (BAL) presentan un amplio metabolismo fermentativo y entre sus sustratos se encuentra el sorbitol. Este polialcohol está presente en plantas, principalmente en las hojas y frutos de la familia *Rosaceae*. Es un compuesto de bajo contenido calórico y una alta hidrofilia, por ello es altamente utilizado en la industria alimentaria y farmacéutica como edulcorante, humectante y estabilizante. Su producción a nivel industrial se lleva a cabo principalmente mediante la reducción química de la glucosa, aunque en los últimos años ha habido trabajos de ingeniería metabólica donde se han utilizado BAL como factorías celulares para la producción de sorbitol a partir de otras fuentes de carbono. Además, la absorción del sorbitol en el intestino es lenta, por lo que la ingesta de altas dosis puede provocar diarreas. También, pueden darse procesos de intolerancia que se ven agravados en pacientes con enfermedades intestinales como gastroenteritis, celiaquía o enfermedades inflamatorias intestinales. Por ello, el uso de bacterias que sean capaces de metabolizar el sorbitol puede tener utilidad probiótica.

En bacterias, el sorbitol es transportado y fosforilado a sorbitol-6-P mediante un sistema de transporte dependiente de fosfoenolpiruvato (PTS), específico para el sorbitol denominado PTS^{gut}. Los genes que codifican los componentes de este sistema, así como los genes involucrados en el metabolismo del sorbitol y su regulación constituyen el operón *gut*. El análisis del genoma de la bacteria *Pediococcus parvulus* 2.6 productora de un (1,3)-β-D-glucano ramificado en posición *O*-2 (β-glucano) (Capítulo 2 de esta tesis) mostró la presencia de este operón en su genoma. El operón *gut* está compuesto de seis genes (*gutFRMCBA*) que se encuentran bajo el control de una única posible región promotora. El gen *gutF* codifica una sorbitol-6-P deshidrogenasa, capaz de transformar el sorbitol-6-P en frutosa-6-P. Los genes *gutR* y *gutM* codifican sendas proteínas reguladoras. Y los genes *gutC*, *gutB* y *gutA* codifican los componentes EIIC, EIIBC y EIIA del sistema de transporte PTS^{gut}. Así, en este trabajo se abordó el estudio del metabolismo del sorbitol por *P. parvulus* 2.6, su influencia en la síntesis del β-glucano, la localización génica del operón y su regulación molecular mediada por las proteínas GutR y GutM.

El estudio metabólico realizado tanto en *P. parvulus* 2.6 como en su estirpe isogénica no productora de β-glucano 2.6NR revelo que: (i) la estirpe 2.6 es capaz de transportar y

metabolizar el sorbitol, de manera más eficiente en co-metabolismo con la glucosa, en un ambiente ácido y en condición de aireación, donde la acción de una NADH oxidasa puede favorecer la formación de NAD^+, cofactor necesario para la conversión de sorbitol-6-P a fructosa-6-P; (ii) posiblemente la glucosa ejerce una represión catabólica del metabolismo del sorbitol, iniciando la estirpe 2.6 el transporte y metabolismo del sorbitol cuando ha consumido toda la glucosa del medio y (iii) la estirpe 2.6NR es incapaz de transportar y metabolizar el sorbitol.

La utilización del método inmunológico, desarrollado en nuestro laboratorio para la detección específica y cuantificación del β-glucano producido por *P. parvulus* 2.6 (Capítulo 1 de esta tesis), mostró que esta bacteria es capaz de utilizar el sorbitol como precursor para la síntesis del β-glucano, con una eficacia similar a la obtenida a partir de la glucosa. En *P. parvulus* 2.6 se ha descrito la existencia de una enzima glucosa-6-P isomerasa que cataliza la reacción reversible de conversión de fructosa-6-P y glucosa-6-P (Velasco et al., 2007), lo que relaciona la ruta catabólica del sorbitol con la ruta biosintética del β-glucano a partir de UDP-glucosa.

P. parvulus 2.6 contiene tres plásmidos naturales denominados pPP1 (39,1 kpb), pPP2 (24,5 kpb) y pPP3 (12,7 kpb). *P. parvulus* 2.6NR fue generada a partir de la cepa 2.6 por tratamiento con bromuro de etidio y novobiocina (Fernández et al., 1995), que produjo la pérdida del plásmido pPP2, portador del gen *gtf* que codifica la GTF glicosiltransferasa responsable de la síntesis del β-glucano. Un análisis de hibridación Southern permitió determinar que el operón *gut* está localizado en el plásmido pPP1 de la cepa 2.6, pero no en el plásmido pPP1* de la cepa 2.6NR. El análisis de las secuencias de ambos plásmidos mostró que una región de 9.473 pb, que contiene el operón *gut*, no está presente en el plásmido pPP1* de 2.6NR. Flanqueando esta secuencia se detectaron genes codificantes de una transposasa y una serin-recombinasa que podrían haber estado involucradas en la pérdida del operón *gut*. En consecuencia, nuestra hipótesis actual de trabajo es que durante el proceso de curación del plásmido pPP2 también sucedieron procesos de recombinación e intercambio génico entre los plásmidos generando la cepa 2.6NR portadora de pPP1*. Sin embargo, para validar esta hipótesis es necesario realizar más trabajos.

Finalmente, nos propusimos estudiar la regulación de la expresión del operón *gut* de *P. parvulus* 2.6 e inferir la función de las dos proteínas reguladoras GutR y GutM. Previamente, la regulación del operón *gut* fue estudiada en *Lactobacillus casei* BL23 por Alcántara y cols. (2008). Los resultados obtenidos por estos autores demostraron que GutR actúa como un activador transcripcional, mientras que la función de GutM era incierta, aunque sí se observó que la proteína era necesaria para la expresión del operón. La baja homología de los operones *gut* de *P. parvulus* 2.6 y *L. casei* BL23 y de sus secuencias operadoras localizadas en las regiones de los promotores transcripcionales, nos hizo asumir que no debería existir complementación entre los sistemas de utilización del polialcohol en estas BAL. Además, detectamos un operón *gut* en la estirpe *Lactobacillus plantarum* 90, cuyos productos proteicos presentaban una homología del 95-100 % con las proteínas codificadas por el operón de *P. parvulus* 2.6, y los promotores transcripcionales con un 99% de identidad. De este modo, nos planteamos realizar el estudio de la regulación del operón *gut* de *P. parvulus* 2.6 tanto en *L. casei* BL23 como en *L. plantarum* 90, clonando distintas regiones de ADN en el vector pRCR (construido en el Capítulo 3 de esta tesis) y analizando la expresión de la proteína fluorescente mCherry. En primer lugar, se clonó la posible región promotora P_{gut} obteniéndose el vector pRCR16, y a partir de esta construcción se clonó bajo su control el gen *gutR*, el gen *gutM* o ambos genes obteniéndose, respectivamente, los plásmidos recombinantes pRCR17, pRCR18, pRCR19. Las 4 construcciones fueron clonadas en *Lactococcus lactis* MG1363 y posteriormente transferidas tanto a *L. casei* BL23 como a *L. plantarum* 90. Los resultados obtenidos en *L. casei* BL23 mostraron que no existe complementación entre ambos sistemas, ya que las proteínas reguladoras de BL23 no fueron capaces de activar la expresión de la proteína mCherry codificada por pRCR16, a partir del promotor P_{gut} de *P. parvulus* 2.6. Los estudios de expresión génica en las bacterias portadoras de las otras dos construcciones determinaron que, en presencia del gen *gutR* de la cepa 2.6, la expresión a partir de P_{gut} se activaba, mientras que si el gen *gutM* se encontraba solo no existía activación. Por otro lado, los resultados obtenidos con las cepas derivadas de *L. plantarum* 90 mostraron la complementación entre ambos sistemas, y como se inducía en mayor medida la expresión de la proteína mCherry en presencia del regulador GutR de *P. parvulus* 2.6 en multicopia. En este sistema, la sobreexpresión de GutM en solitario produjo un efecto deletéreo del crecimiento de *L. plantarum* 90 en las primeras fases de inducción. Sin embargo, en la fase tardía de la inducción (a partir de las 5 h) se detectó una mayor expresión de mCherry en presencia de GutM que la producida en presencia de GutR. Por otra parte, tanto en *L. casei* BL23 como en *L. plantarum* 90, la sobreexpresión conjunta de GutR y GutM produjo una inducción más moderada de la expresión de mCherry. Todos estos resultados junto con la predicción de la existencia de una posible proteína fusionada GutR-M, indican que ambas proteínas interactúan entre sí y son necesarias para la regulación de la expresión del operón *gut*. Sin embargo, para probar esta hipótesis será necesaria la realización, en un futuro, de trabajos complementarios.

Mi aportación a este trabajo

Para lograr la consecución de este trabajo científico, mi aportación fue la siguiente. Contribuí a la realización de todo el trabajo experimental, salvo a la secuenciación mediante la técnica de “primer walking” de la región de 11.746 pb en la estirpe 2.6 que contiene el operón *gut*. También realicé el borrador del manuscrito, así como las figuras y tablas que contiene.

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Characterization of the Sorbitol Utilization Cluster of the Probiotic *Pediococcus parvulus* 2.6: Genetic, Functional and Complementation Studies in Heterologous Hosts

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Pediococcus parvulus 2.6 secretes a 2-substituted (1,3)-β-D-glucan with prebiotic and immunomodulatory properties. It is synthesized by the GTF glycosyltransferase using UDP-glucose as substrate. Analysis of the *P. parvulus* 2.6 draft genome revealed the existence of a sorbitol utilization cluster of six genes (*gutFRMCBA*), whose products should be involved in sorbitol utilization and could generate substrates for UDP-glucose synthesis. Southern blot hybridization analysis showed that the cluster is located in a plasmid. Analysis of metabolic fluxes and production of the exopolysaccharide revealed that: (i) *P. parvulus* 2.6 is able to metabolize sorbitol, (ii) sorbitol utilization is repressed in the presence of glucose and (iii) sorbitol supports the synthesis of 2-substituted (1,3)-β-D-glucan. The sorbitol cluster encodes two putative regulators, GutR and GutM, in addition to a phosphoenolpyruvate-dependent phosphotransferase transport system and sorbitol-6-phosphate dehydrogenase. Therefore, we investigated the involvement of GutR and GutM in the expression of *gutFRMCBA*. The promoter-probe vector pRCR based on the *mrfp* gene, which encodes the fluorescence protein mCherry, was used to test the potential promoter of the cluster (P_{gut}) and the genes encoding the regulators. This was performed by transferring by electrotransformation the recombinant plasmids into two hosts, which metabolize sorbitol: *Lactobacillus plantarum* and *Lactobacillus casei*. Upon growth in the presence of sorbitol, but not of glucose, only the presence of P_{gut} was required to support expression of *mrfp* in *L. plantarum*. In *L. casei* the presence of sorbitol in the growth medium and the pediococcal *gutR* or *gutR* plus *gutM* in the genome was required *for* P_{gut} functionality. This demonstrates that: (i) P_{gut} is required for expression of the *gut* cluster, (ii) P_{gut} is subjected to catabolic repression in lactobacilli, (iii) GutR is an activator, and (iv) in the presence of sorbitol, *trans*-complementation for activation of P_{gut} exists in *L. plantarum* but not in *L. casei*.

Keywords: ***Pediococcus parvulus*, exopolysaccharides, β-glucans, sorbitol, lactic acid bacteria, probiotic**

INTRODUCTION

Sorbitol, also named D-glucitol, is a six-carbon sugar polyol widespread in plants, particularly in fruits, such as berries, cherries, plums, pears and apples. However, sorbitol is obtained industrially, by catalytic hydrogenation of glucose or glucose/fructose mixtures. This polyol has a relative sweetness of about 60% compared to that of sucrose, high-water solubility and is largely used as a low calorie sweetener, humectant, texturizer and softener (Zumbé et al., 2001). In addition, sorbitol is used in the production of pharmaceutical compounds, such as sorbose and ascorbic acid, and as a vehicle for drug-suspension (Silveira and Jonas, 2002). Sorbitol has also a potential prebiotic effect *in vivo*, since it does not contribute to the formation of dental caries, is slowly and only partially absorbed in the small intestine and can reach the colon where it can act as substrate for bacterial fermentation. Supplementation with sorbitol resulted in enrichment of lactobacilli in rat colon and cecum (Sarmiento-Rubiano et al., 2007).

Sorbitol absorption is mediated by dose and concentration. Doses greater than 30 g can cause water retention, resulting in osmotic diarrhea, bloating, flatulence, cramping and abdominal pain (Fernández-Bañares et al., 2009). These doses vary depending on the condition of the intestinal absorption surface. In patients with malabsorption, the ingestion of 5–20 g, provoked diarrhea and gastrointestinal complications (Montalto et al., 2013). In the colon, this sugar alcohol is metabolized by some species of *Lactobacillus* and is also a preferred carbon source for human intestinal bifidobacteria (Sarmiento-Rubiano et al., 2007).

Furthermore, utilization of sorbitol as a carbon source has been described in a variety of bacteria within the fila proteobacteria (Yamada and Saier, 1988; Aldridge et al., 1997) and firmicutes (Tangney et al., 1998; Boyd et al., 2000; Yebra and Pérez-Martínez, 2002). Among the firmicutes, there are some lactic acid bacteria (LAB) with catabolic pathways for sorbitol metabolism (Rhodes and Kator, 1999; Sarmiento-Rubiano et al., 2007). These pathways are encoded by genes organized in *gut* operons, and include the sorbitol transport system, sorbitol-6-phosphate dehydrogenase (S6PD) as well as regulatory protein(s), and those of *Lactobacillus casei* and *Lactobacillus plantarum* have been characterized (Nissen et al., 2005; Ladero et al., 2007; Alcantara et al., 2008).

Sorbitol is transported into the cells and phosphorylated to sorbitol-6-phosphate by a phosphopyruvate-dependent phosphotransferase (PTS) sorbitol system (PTS^{gut}). Each PTS is composed of two cytoplasmic enzymes, common to the transport of different compounds (EI and HPr) and of different membrane-associated enzyme complexes (EII), specific for one, or several substrates. The genes *gutC*, *gutB* and *gutA* encode the EII domain of a sorbitol PTS (Alcantara et al., 2008). The *gutF* gene encodes a sorbitol-6-P dehydrogenase, which catalyzes the conversion of sorbitol-6-phosphate to fructose-6-phosphate, a compound that is introduced into the glycolytic pathway with NADH regeneration (Nissen et al., 2005). The *gutR* and *gutM* genes encode two regulatory proteins. The role of the GutM and GutR proteins has been studied in *Escherichia coli*, operating GutM as an activator and GutR as a repressor (Yamada and Saier, 1988). In the firmicutes group, the analyzed *gut* operons contain homologs to the *gutM* and *gutR* genes, but the role of GutR regulator is different from that of *E. coli*. The GutR of *L. casei* has been functionally characterized and it has been shown to be a PTS-controlled transcriptional activator, via a PTS regulation binding domain (PRD) (Stülke et al., 1998). Also, both the GutR binding sequence and the PRD domain are conserved in firmicutes. GutM encodes a highly conserved protein in firmicutes and in *L. casei* plays a regulatory role (Alcantara et al., 2008).

Pediococcus parvulus 2.6 (Werning et al., 2006) (previously named *Pediococcus damnosus*) is a lactic acid bacteria isolated from a ropy cider (Fernández et al., 1995). This LAB produces a 2-substituted (1,3)-β-D-glucan exopolysaccharide (EPS) (Dueñas-Chasco et al., 1997), with high molecular mass ($>10^6$ Da), and whose rheological properties showed its potential utility as a biothickening agent (Velasco et al., 2009). The presence of this EPS improves some probiotic features of *P. parvulus* 2.6, including tolerance to simulated gastrointestinal conditions and adherence to Caco-2 cell lines and reduces inflammation-related cytokine levels produced by polarized macrophages (Fernández de Palencia et al., 2009; Immerstrand et al., 2010). Moreover, the purified EPS improves the growth, viability and adhesion capability of probiotic microorganisms (Russo et al., 2012), also it activates macrophages with anti-inflammatory effects (Notararigo et al., 2014), and decreases the levels of the proinflammatory IL8 in human intestine cultures (Notararigo et al., unpublished data). The draft genome of *P. parvulus* 2.6 has been determined (Pérez-Ramos et al., 2016), and its analysis showed the existence of a putative sorbitol utilization *gut* operon in this bacterium. Thus, this current work focuses on the genomic location, expression and metabolic involvement of the *gut* operon of *P. parvulus* 2.6 in sorbitol catabolism, as well as its interplay with EPS production by this bacterium.

MATERIALS AND METHODS

Bacterial Strains and Growth Conditions

The bacteria used in this work are listed in **Table 1**. *Pediococcus* and *Lactobacillus* strains were routinely grown in de Man Rogosa Sharpe (MRS) broth (Pronadisa, Madrid, Spain) at 30°C and 37°C, respectively. *Lactococcus lactis* strains were grown in ESTY broth (Pronadisa) supplemented with 0.5% glucose at 30°C. When bacteria carried the pRCR plasmid or its derivatives the medium was supplemented with chloramphenicol (Cm) at 5 μg mL^{-1} for *L. lactis* and at 10 μg mL^{-1} for lactobacilli. *E. coli* V517 was grown in LB broth and incubated at 37°C.

For evaluation of sorbitol utilization, *P. parvulus* strains were grown in a MRS broth made by components (de Man et al., 1960) without glucose, pH was adjusted to 5.2 and the medium supplemented with 10 mM glucose (MRSG), 30 mM sorbitol (MRSS) or 10 mM glucose plus 30 mM sorbitol (MRSGS) at 30°C. Prior selection of conditions for growth in presence of sorbitol several tests were performed. First various carbon sources were tested (10 mM glucose, 10 mM fructose or 10 mM maltose) and

TABLE 1 | Bacteria used in this work.

Bacteria	Plasmid	Resistance	Characteristics	Reference
Pediococcus parvulus 2.6	pPP1, pPP2, and pPP3	–	2-substituted (1,3)-β-D-glucan producer	Pérez-Ramos et al., 2016
P. parvulus 2.6NR	pPP1 and pPP3	–	Non-EPS-producing strain. Derivative of 2.6 strain by pPP2 plasmid curing	Fernández et al., 1995
Lactococcus lactis subsp. *cremoris* MG1363	–	–	Plasmid free type strain used for plasmid cloning	Wegmann et al., 2007
L. lactis subsp. *cremoris* MG1363[pRCR]	pRCR	Cm^R	Source of promoter probe pRCR containing the *mrfp* gene, which encodes the fluorescent mCherry protein	Mohedano et al., 2015
Escherichia coli V517	8 plasmids pVA517A through pVA517H	ND	Source of plasmids used as references in agarose gel analysis	Macrina et al., 1978
Lactobacillus casei BL23	–	–	Bacteria used for heterologous gene expression	Mazé et al., 2010
L. casei BL23[pRCR16]	[pRCR16]	Cm^R	Derivative of pRCR by cloning of P_{gut} upstream of *mrfp*	This study
L. casei BL23[pRCR17]	[pRCR17]	Cm^R	Derivative of pRCR16 by cloning of *gutR* upstream of *mrfp*	This study
L. casei BL23[pRCR18]	[pRCR18]	Cm^R	Derivative of pRCR16 by cloning of *gutM* upstream of *mrfp*	This study
L. casei BL23[pRCR19]	[pRCR19]	Cm^R	Derivative of pRCR16 by cloning of *gutMR* upstream of *mrfp*	This study
Lactobacillus plantarum 90	1 uncharacterized plasmid	ND	Bacteria used for heterologous gene expression	Lamontanara et al., 2015
L. plantarum 90[pRCR16]	[pRCR16]	Cm^R	Derivative of pRCR by cloning of P_{gut} upstream of *mrfp*	This study
L. plantarum 90[pRCR17]	[pRCR17]	Cm^R	Derivative of pRCR16 by cloning of *gutR* upstream of *mrfp*	This study
L. plantarum 90[pRCR18]	[pRCR18]	Cm^R	Derivative of pRCR16 by cloning of *gutM* upstream of *mrfp*	This study
L. plantarum 90[pRCR19]	[pRCR19]	Cm^R	Derivative of pRCR16 by cloning of *gutMR* upstream of *mrfp*	This study

ND, no determined; Cm^R, resistance to chloramphenicol.

pH at 6.8, 5.2 or 4.0 and then influence of aeration was evaluated in presence of 10 mM glucose at either pH 6.8 and 5.2 (results not show).

For evaluation of mCherry expression, *Lactobacillus* strains were grown in a MRSG containing 55 mM (1% w/v) glucose or in a MRSS containing 55 mM (1% w/v) sorbitol at 37°C.

Plasmidic DNA Preparations

Total plasmidic DNA preparations of *P. parvulus* 2.6 and 2.6NR strains were prepared as follows. Bacterial cultures were grown to an optical density at 600 nm ($OD_{600\ nm}$) of 2.5, and 100 mL of each culture were sedimented by centrifugation at 10,000 × *g* for 20 min at 4°C. The cells were resuspended in 4 mL of a solution containing 50 mM Tris/HCl pH 8.0, 10 mM EDTA, lysozyme (30 mg mL^{-1}) and RNasa A (10 μg mL^{-1}), and incubated for 30 min at 37°C. Then, 4 mL of a solution containing 220 mM NaOH and 1.33% sodium dodecyl sulfate) were added and samples were incubated for 5 min at room temperature. Upon addition of 5 M potassium acetate pH 5.0 (4 mL), samples were centrifugated at 10,000 × *g* for 15 min at 21°C. The DNA present in the supernatants was precipitated, concentrated by addition of 8.7 mL of isopropanol, sedimented by centrifugation at 10,000 × *g* for 15 min at 4°C, and resuspended in 10 mM Tris, 1 mM EDTA buffer (4.3 mL). The DNA preparation was deproteinated by treatment with 7.5 M ammonium acetate (2.7 mL) and phenol (4.3 mL) during 5 min at room temperature and then sedimented at 10,000 × *g* for 5 min at 21°C. The aqueous phase containing total plasmidic DNA was further purified by isopycnic CsCl density gradient centrifugation and dialysis as previously described (López et al., 1989). The final recovery was 54 μg and 58 μg for 2.6 and 2.6NR DNA preparations, respectively.

The recombinant plasmids from the lactococcal and lactobacilli strains were isolated using the High pure plasmid isolation kit (Roche) as follows. Bacteria were grown until stationary phase (10^9 colony forming units mL^{-1}) and 1 mL of each culture were sedimented by centrifugation at 10,000 × *g* for 10 min at 4°C. Cells were resuspended in solution I of the kit supplemented with lysozyme (30 mg mL^{-1}) and were incubated for 30 min at 37°C. Then, plasmid isolation were performed as described in the kit protocol, eluting the plasmidic DNA in 100 μL at approximately 100 ng μL^{-1}.

Sequencing

DNA sequencing was performed by the dideoxy method at Secugen (Madrid, Spain). The sequencing of the sorbitol utilization cluster and the flanking regions of pPP1 of *P. parvulus* 2.6 was performed using total plasmidic DNA preparations of the bacterium (see above) with the walking strategy and the sequence has been deposited in GenBank (accession No MF766019). The lack of sorbitol cluster in the 2.6NR strain was confirmed by sequencing of its pPP1 plasmid by using as substrates a total plasmidic preparation of 2.6NR strain and either pPP1*F or pPP1*R primers (see **Table 2**) In addition, in the case of sequencing with pPP1*F, it was also used as substrate the product of a polymerization reaction catalyzed by the bacteriophage Φ29 DNA polymerase with plasmidic DNA of *P. parvulus* 2.6NR and hexamers containing random sequences.

Construction of pRCR16, pRCR17, pRCR18, and pRCR19

A region located upstream of the *P. parvulus* 2.6 *gut* operon carrying the putative P_{gut} promoter and the *gutR* and *gutM* genes was cloned into the promoter probe pRCR vector. To this end, three DNA regions of pPP1 plasmid were amplified with Phusion High Fidelity Polymerase (PHFP, ThermoFisher Scientific) by using a plasmidic DNA preparation of *P. parvulus* 2.6 and the primers depicted in **Table 2**, which have homology with pPP1 DNA and carry restriction sites suitable for cloning. Plasmid pRCR16 (**Figure 1**) was generated by ligation of the P_{gut} promoter to the pRCR promoter probe vector (Mohedano et al., 2015), after double digestion of both DNAs with BglII and XmaI (New England Biolabs, Ipswich, MA, United States), with the T4 DNA ligase (New England Biolabs). Then, between the XmaI and XbaI restriction sites of pRCR16 three amplicons were independently cloned, containing *gutR*, *gutM* or *gutRM,* generating plasmids pRCR17, pRCR18 and pRCR19, respectively. The clonings were performed in *L. lactis* MG1363, the ligations mixtures were used to transform the bacteria by electroporation (25 μF, 2.5 kV and 200 Ω in 0.2 cm cuvettes), as previously described (Dornan and Collins, 1987) and transformants were selected in ESTY-agar plates supplemented with Cm at 5 μg mL^{-1}. The inserts present in the new four recombinant plasmids were confirmed by automated sequencing. Then, DNA preparations of pRCR17, pRCR18 and pRCR19 obtained from *L. lactis* MG1363 (0.5 μg) were used for transfer to lactobacilli by electroporation (25 μF, 1.3 kV and 200 Ω in 0.1 cm cuvettes) as previously described (Berthier et al., 1996) and transformants were selected in MRSG-agar plates supplemented with Cm at 10 μg mL^{-1}.

Southern Hybridization

Plasmid samples 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^{-1}. The image of the gels was obtained with GelDoc 200 (BioRad) and the bands were quantitated with the Quantity One 4.5.2 software (BioRad). The DNA fragments were transferred to a nylon membrane Biodyne A (PALL Gelman Laboratory, AnnArbor, MI, United States) by 5 inches Hg of vacuum for 2 h using the Vacuum Blotter model 785 (Bio-Rad). Internal regions of *gutF*, *gutR* and *gutB* genes were amplified by PCR generating amplicons 1, 2, and 3, respectively, in reactions catalyzed by PHFP, and by using as substrate total plasmidic DNA preparation of *P. parvulus* 2.6 and the primer pairs shown in **Table 2**. Then, the amplicons were labeled with digoxigenin-dUTP by using the DIG high prime DNA labeling and detection starter kit II (Roche, Mannheim, Germany). Each DIG-labeled DNA probe (25 ng mL^{-1}) was used for hybridization at 45°C following the specifications of the kit's supplier. The hybridization bands were revealed with the chemiluminescent substrate CSPD, and the signals were detected

TABLE 2 | Oligonucleotides used in this work.

Primers	Sequence (5′- 3′)	Utilization	Amplicon size (bp)
pts1F	TGCGGAAGCGGTTAATCGGCT	*gutF* DNA probe[a]	618
pts1R	CCACGACTCTTGCCTCCCGCA		
ptsRF	CGAACTGGAAGCAACCTGGGA	*gutR* DNA probe[a]	652
ptsRR	CCGATGAATAATTGGCGCTGC		
ptsBF	GGAATGGAAGCTGTTGATGGC	*gutB* DNA probe[a]	643
ptsBR	CAACGCCAATCAAGGTCCCGA		
pgutBglIIF	GAAGATCTACCATATGGCGATAATGAAAA	Cloning of P_{gut} in pRCR[a]	186
pgutXmaIR	TCGCTCCCGGGTCATTTCTTTTC		
gutRXmaIF	CGTGGTTAACCCGGGAATTTTAGTTG	Cloning of *gutR* in pRCR16[a]	1952
gutRXbaIR	GCTCTAGAAACGCACTGACTAGGATCA		
gutMXmaIF	TCCCCCCGGGTTAAATCAGTTGATGGA	Cloning of *gutM* in pRCR16[a]	597
gutMXbaIR	GCTCTAGAACAGCCCATAAAGCCC		
gutRXmaIF	CGTGGTTAACCCGGGAATTTTAGTTG	Cloning of *gutRM* in pRCR16[a]	2472
gutMXbaIR	GCTCTAGAACAGCCCATAAAGCCC		
pPP1*F	CATAGTTCACTGGGCTACCA	Sequencing of pPP1*[b]	–
pPP1*R	TAGCGGTGCCTCCCTTTAAT		–

[a] *Plasmidic DNA preparations of P. parvulus 2.6 was used as substrate for the PCR reactions.* [b] *Plasmidic DNA preparations of P. parvulus 2.6NR was used as substrate for the DNA sequencing.*

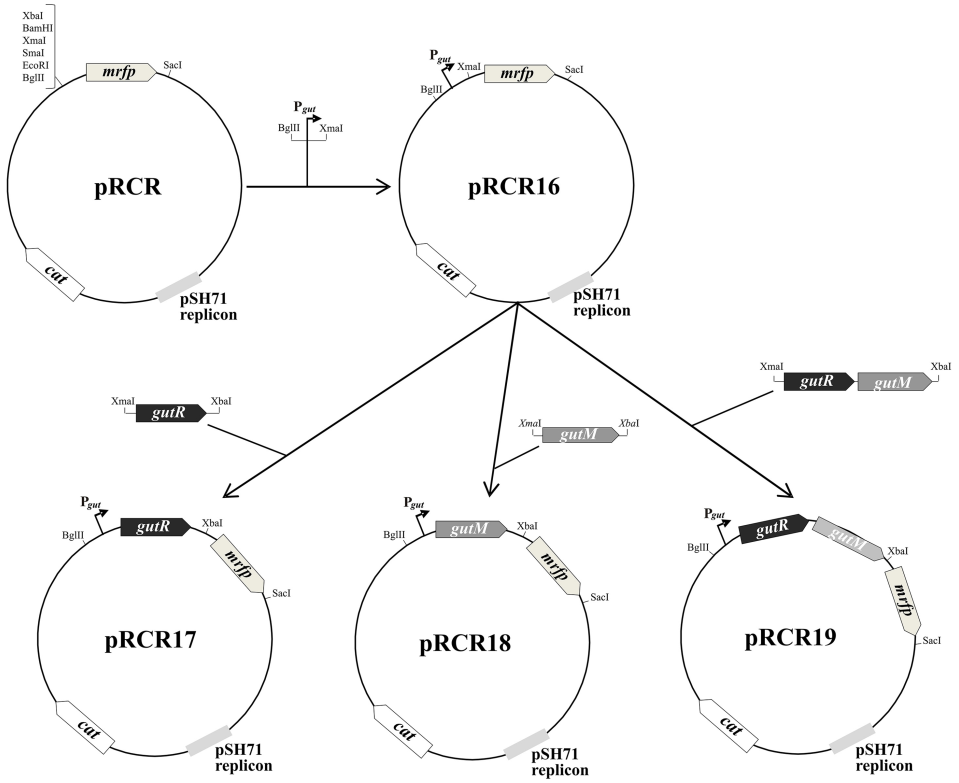

FIGURE 1 | Scheme of construction of plasmids pRCR16, pRCR17, pRCR18, and pRCR19. Maps of these plasmids and of the parental pRCR are depicted. The pertinent restriction sites are shown, as well as the putative promoter of the *gut* operon (P_{gut}) and the following genes: *mrfp, cat*, *gutR* and *gutM* encoding the mCherry, a chloramphenicol acetyltransferase, GutR and GutM, respectively.

with the LAS-3000 imaging system (Fujifilm, Stamford, CT, United States).

Analysis of the Metabolic Fluxes of *P. parvulus* and Its EPS Production

P. parvulus 2.6 and 2.6NR strains were grown in either MRSG or MRSGS under aerobic conditions (shaking at 180 rpm), at 30°C during 66 h, and samples were taken at the times indicated in **Figure 2** to monitor growth by determination of optical density at 600 nm and of acidification of the media by measuring pH. Also, samples were centrifuged at 16,000 × *g* for 30 min at 4°C, and the levels of glucose, sorbitol, lactic acid and EPS in the supernatants were analyzed. The experiments were performed in triplicate for each strain and in each condition of growth.

Analysis of Culture Supernatants by Gas Chromatography-Mass Spectrometry (GC-MS)

The concentration of glucose, sorbitol and lactic acid was determined by GC–MS using myo-inositol as internal standard. For this analysis, *myo*-inositol (100 μg) was first added to aliquots of the bacterial culture supernatants. The mixture was lyophilized and derivatized with 2.5% hydroxylamine chloride in pyridine for 30 min at 70°C, to form the sugar oximes. Afterward, *bis*-trimethylsilyl trifluoroacetamide (BSTFA) was added and samples were incubated for 45 min at 80°C, to form the trimethylsilylated derivatives. Identification and quantification of the compounds were performed by GC–MS on a 7980A-5975C instrument (Agilent, Santa Clara, CA, United States) equipped with a HP-5MS column (30 m × 0.25 mm I.D. × 0.2 μm film thickness) with helium as the carrier gas. Injector and detector were set at 275°C. Samples (1 μL) were injected with a split ratio of 1:50 with a temperature program: 80°C for 4 min, then 15°C min^{-1} to 270°C and finally 30°C min^{-1} to 310°C (2 min). The peaks in the chromatograms corresponding to sugars and lactic acid were identified by their retention times. Quantifications were calculated using the peak areas and the calibration standard curve for each compound.

Quantification of the 2-Substituted (1,3)-β-D-Glucan Produced by *P. parvulus*

A competition (ELISA) method for the specific detection of the EPS synthesized by *P. parvulus* 2.6, based on *Streptococcus pneumoniae* serotype 37 antibodies, was performed as previously described (Werning et al., 2014). Briefly, the ELISA assay was carried out in 96-Well Nunc-Immuno MicroWell MaxiSorp plates (Thermo Fisher Scientific), and the EPS of *P. parvulus* 2.6, purified as previously described (Notararigo et al., 2013), was immobilized in each well (62.5 ng per well).

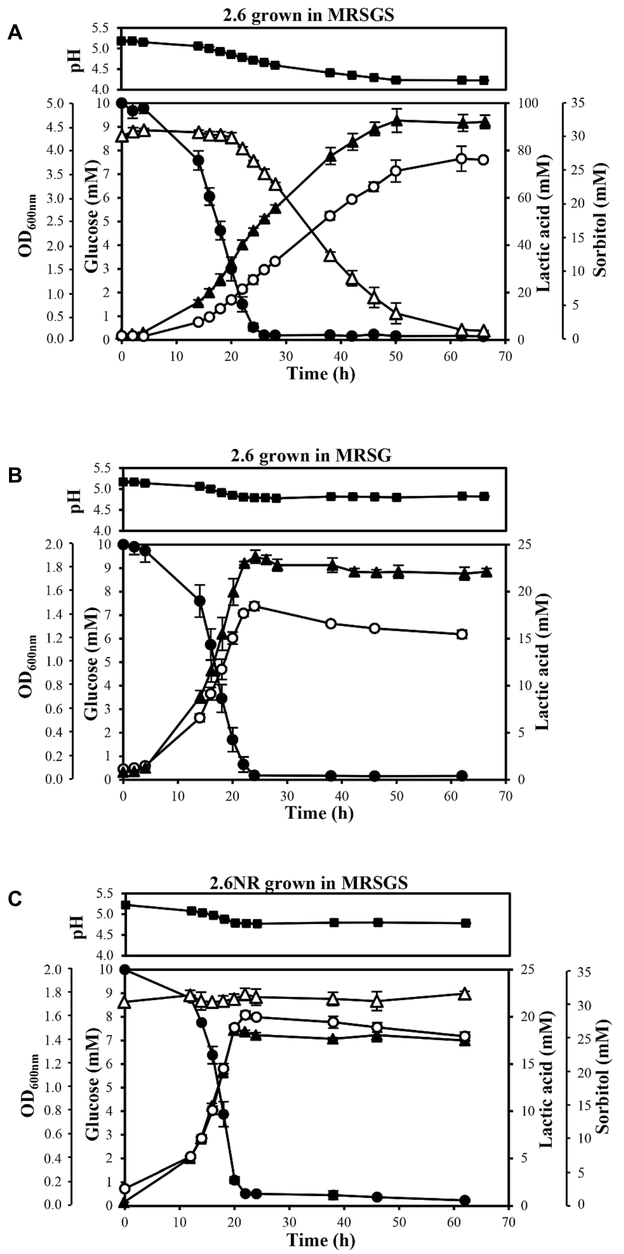

FIGURE 2 | Analysis of metabolism of *P. parvulus* 2.6 **(A,B)** and 2.6NR **(C)** strains. Bacteria were grown in the indicated media. Symbols: OD$_{600\ nm}$ (▲), glucose (●), sorbitol (△), lactic acid (○) and pH (■). The experiments were performed in triplicate and the mean value and standard deviation is depicted.

Culture supernatants [diluted with phosphate-buffered saline (PBS) pH 7.2 when necessary] were used as competitor for binding to the primary antibody (dilution 1:800 of anti-serotype 37, Statens Serum Institut, Copenhagen, Denmark). Then, primary antibody was conjugated with a secondary antibody, polyclonal Anti-Rabbit IgG alkaline phosphatase (Sigma–Aldrich, Saint Louis, MO, United States) diluted 1:25,000, and finally was revealed with *p*-nitrophenylphosphate in diethanolamine buffer (Sigma–Aldrich). Reaction signals were detected with a microtiter plate reader model 680 (Bio-Rad, Hercules, CA, United States), measuring the OD at 415 nm. Quantification was performed using a standard curve generated by the competition for the primary antibody of serial dilutions of the purified *P. parvulus* 2.6 EPS dissolved in PBS.

Detection of mCherry Fluorescence in LAB Carrying pRCR16, pRCR17, pRCR18, or pRCR19

To detect the expression levels of the mCherry fluorescent protein, *L. plantarum* Lp90 strains carrying the pRCR derivatives were diluted 1:100 and grown in MRS supplemented with 1% glucose in static mode at 37°C, until mid-exponential phase. Then the cultures were centrifuged at 9,000 × *g* for 10 min at room temperature, and the cells were washed with one volume of PBS pH 7.2 prewarmed at 37°C. Then, the bacteria were resuspended in the same volume of MRS broth supplemented with 1% sorbitol or 1% glucose prewarmed at 37°C. Cultures were incubated at 37°C with agitation of 180 rpm, and samples were taken each hour. Two hundred microliter of all chilled samples were centrifuged at 9,000 × *g* for 10 min at 4 °C and cells were washed once with chilled PBS buffer pH 7.2. Samples were resuspended in 200 μL of PBS buffer pH 7.2 and used to measure the fluorescence levels of mCherry protein in a 96-Well Nunc U96 MicroWell plate (Thermo Fisher Scientific) in a Varioskan Flash equipment (Thermo Fisher Scientific), using 587 and 610 nm wavelengths for excitation and detection of emission, respectively. In addition, appropriate dilutions were prepared to estimate culture biomass by measuring the OD$_{600\ nm}$. Three independent trials were performed and the same fresh suspensions, without fixing, were used for phase contrast and fluorescent microscopy analysis with a Leica DM1000 model microscope (Leica Microsystems, Mannheim, Germany) with a light source EL6000 and a filter system TX2 ET for detection of red fluorescence. The microscope was connected to a DFC3000G camera (Leica Microsystems) with a CCD sensor. Image analysis was performed using Leica Application Suite X Software (Leica Microsystems).

To detect the expression of the mCherry fluorescent protein, *L. casei* BL23 strains carrying the pRCR derivatives were grown and processed in the same manner as the *L. plantarum* cultures, except that preinoculum cultures were diluted in MRS supplemented with 1% glucose or 1% sorbitol to an OD$_{600\ nm}$ = 0.1 and then were incubated at 37°C with agitation of 180 rpm for 16 h, until they reached early stationary phase. Then, 1 mL of each culture was centrifuged and washed with PBS as above. Samples were concentrated five-fold and used to measure the fluorescence levels and to take fluorescence images as described above.

Bioinformatic Analysis

The DNA sequence of plasmid pPP1 was analyzed with the programs included in the DNASTAR Lasergene 12 (DNAstar Inc. Madison, WI, United States). Homologies of pPP1 DNA

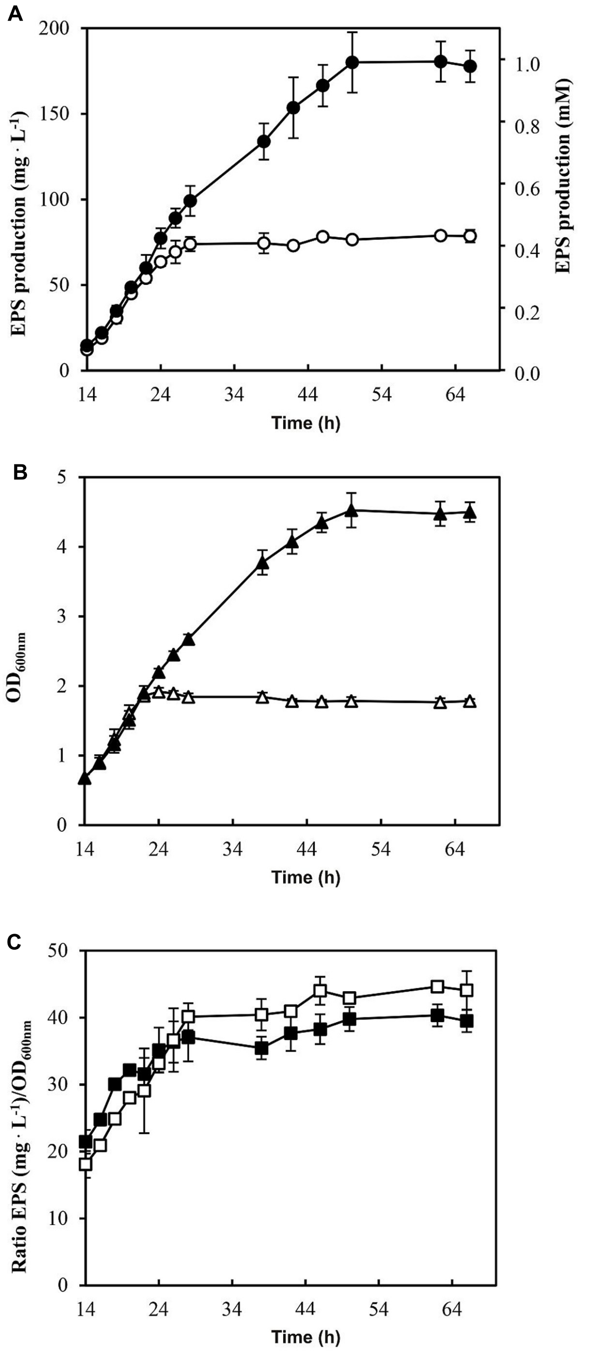

FIGURE 3 | Analysis of EPS production by *P. parvulus* 2.6 in MRSGS (●), (▲) and (■) and in MRSG (○), (△) and (□). **(A)** Concentration of EPS present in cultures supernatants is depicted. The results were expressed in mg of EPS per L or in mmol of glucose per L. **(B)** The $OD_{600\ nm}$ of the cultures is depicted. **(C)** Specific EPS concentration is shown and it was calculated as the ratio EPS concentration/$OD_{600\ nm}$. The experiments were performed in triplicate and the mean value and standard deviation is depicted

sequences and of its inferred translated products with the NCBI data bases of the National Center for Biotechnology Information (NCBI) were analyzed with the Basic Local Alignment Search Tool (BLAST)[1]. Multiple sequence alignment of genes and proteins were performed with Clustalx 2.1[2] programs.

Transmembrane helices in GutM were predicted using TMHMM 2.0[3] (TMpred[4]) programs. Prediction of secondary structures in the gut mRNA was accomplished with the mfold 2.3 program[5].

RESULTS

Analysis of *P. parvulus* Sorbitol Metabolism

Sorbitol could be a substrate for the synthesis of *P. parvulus* 2.6 EPS and analysis of the DNA sequence of the draft genome of this bacterium (Pérez-Ramos et al., 2016) with the BLAST program revealed a putative *gut* operon, that could be involved in transport and catabolism of this compound. Therefore, growth of *P. parvulus* 2.6 and its isogenic EPS-non-producing (non-ropy) 2.6NR strain in MRS (without glucose) and MRSS (medium containing sorbitol) was tested. The 2.6NR strain showed the same poor growth in both media (Supplementary Figure S1). However, the presence of sorbitol in the medium significantly improved the growth of the 2.6 strain (Supplementary Figure S1), reaching a final $OD_{600\ nm}$ of 3.0 in MRSS *versus* 0.45 in MRS, indicating that this bacterium was able to utilize sorbitol. Nevertheless, the growth of *P. parvulus* 2.6 in MRSS was very slow and took more than 12 days to reach the final optical density (Supplementary Figure S1). Therefore, in order to improve the growth rate of 2.6 strain, the influence of modifying various parameters in bacterial growth in MRSS was investigated. The best inferred conditions were the usage of a MRSGS containing as carbon sources 10 mM glucose plus 30 mM sorbitol, pH = 5.2, and growth with aeration at 30°C. Thus, these conditions were used to investigate a potential interplay between sorbitol utilization and EPS production by *P. parvulus* 2.6.

A comparative study of the metabolic fluxes of *P. parvulus* strains by analysis of culture supernatants during growth in MRSG or MRSGS corroborated that 2.6, but not 2.6NR, was able to ferment sorbitol (**Figure 2**). Co-metabolism of sorbitol and glucose by the 2.6 strain resulted in an increase of 2.5-fold in the final biomass estimated by the $OD_{600\ nm}$ of the cultures. Values of 4.48 ± 0.18 in MRGS (**Figure 2A**) compared to 1.77 ± 0.06 reached in MRSG (**Figure 2B**), the latter being similar to 1.36 ± 0.05 observed for the 2.6NR strain in MRGS (**Figure 2C**). In addition, a prolonged exponential growth phase of the 2.6 strain was observed in the MRSGS medium (50 h *versus* 20 h, **Figures 2A,B**). In the 2.6NR culture supernatants, the initial sorbitol levels (30 mM) remained constant during the entire time period of the assays, revealing that this bacterium was unable to transport sorbitol to the cytosol (**Figure 2C**). Moreover, the analysis of the carbon source consumption by the 2.6 strain

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[1] https://blast.ncbi.nlm.nih.gov/Blast.cgi

[2] http://www.ebi.ac.uk/Tools/msa/clustalw2/

[3] http://www.cbs.dtu.dk/services/TMHMM-2.0/

[4] http://embnet.vital-it.ch/software/TMPRED_form.html

[5] http://unafold.rna.albany.edu/?q=mfold/RNA-Folding-Form

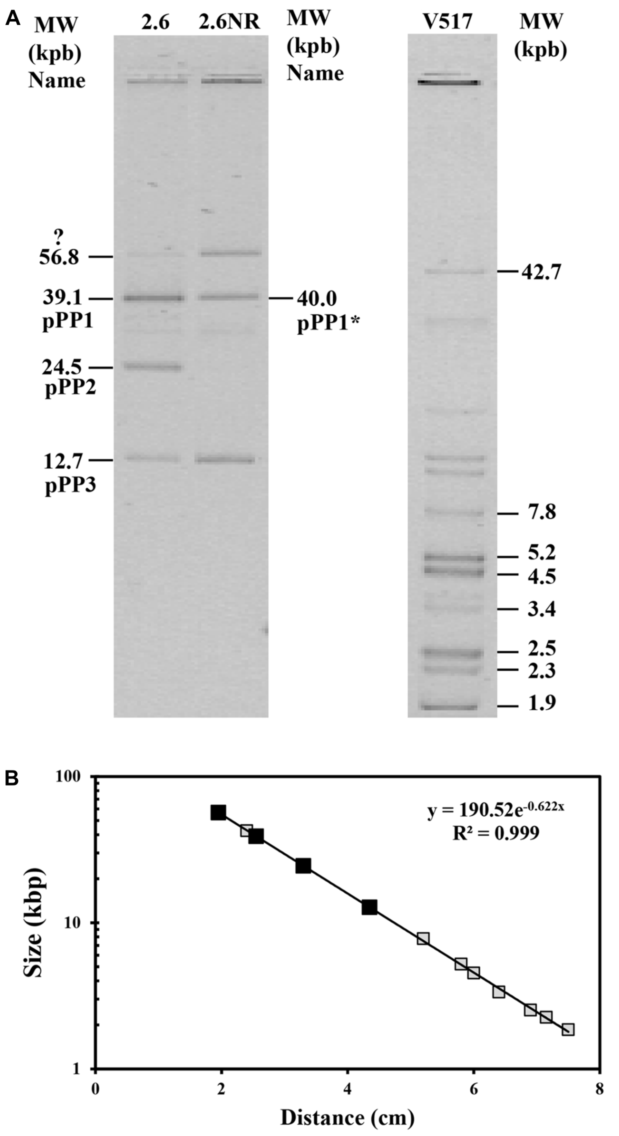


FIGURE 4 | Detection of plasmids of *P. parvulus* 2.6 and 2.6NR strains. Plasmids preparations of *P. parvulus* strains and of *E. coli* V517 were analyzed in a 0.7% agarose gel. **(A)** The image of the gel. The three samples were run in the same gel. In **(B)** the calibration curve for plasmid size determination is depicted. Symbols: plasmids from *E. coli* V517 (□), plasmids from *P. parvulus* strains (■).

showed that glucose started to be transported to the cytosol after 2 h of growth, and upon 26 h of incubation the monosaccharide was undetectable in the culture supernatants (**Figures 2A,B**). Furthermore, only after 20 h of incubation did the 2.6 strain start to internalize the sorbitol and presumably to metabolize it, because the bacterium did not enter into the stationary phase until the sorbitol was consumed (**Figure 2A**). The metabolic activity of the two strains was monitored by detecting the lactic acid production, since it is the main metabolic end-product because pediococci are homofermentative bacteria. The results showed that the 2.6 strain grown in MRSG (**Figure 2B**) and the 2.6NR strain grown in MRSGS (**Figure 2C**) released to the culture media similar amounts of lactic acid, the maximum levels being 18.45 ± 0.45 mM and 20.19 ± 0.42 mM, respectively. By contrast, the 2.6 strain grown in the presence of both carbon sources showed a higher lactic acid production, up to 76.03 ± 0.43 mM (**Figure 2A**). Correlating with these results, the final pH of the 2.6 cultures in MRSG and of the 2.6NR cultures in MRSGS was similar (4.81 ± 0.02 *versus* 4.78 ± 0.02), and higher than that of the 2.6 cultures in MRSGS (4.23 ± 0.02).

Furthermore, the EPS production by *P. parvulus* 2.6 in the presence or absence of sorbitol was investigated. Significant EPS levels were detected after 14 h of growth in MRSG and MRSGS media. Therefore, the data depicted in **Figure 3** correspond to those obtained within the 14–62 h incubation period. The results revealed that the bacterium produced EPS during the growth in MRSGS and synthesized higher levels of the polymer in this medium than in MRSG (**Figure 3A**). Thus, after 62 h of growth in MRSG, the 2.6 strain produced 78.6 ± 3.7 mg L^{-1} of EPS, while in MRSGS synthesized 180.5 ± 11.8 mg L^{-1}. Additionally, in order to evaluate the specific efficiency of the EPS production depending on the carbon source used, the ratio between EPS concentration and the biomass estimated from the $OD_{600\ nm}$ (**Figure 3B**) was calculated (**Figure 3C**). The results showed that irrespectively of the carbon source, the bacteria had almost identical efficiency of EPS production, which increased during the exponential and stationary phases of growth (**Figure 3C**).

Determination of Genomic Location of the *gut* Operon

P. parvulus 2.6 probably carries three natural plasmids, which were previously named pPP1, pPP2 and pPP3 (Werning et al., 2006), and we have identified only three plasmid replication machineries in the *P. parvulus* draft genome (Pérez-Ramos et al., 2016). In addition, the *P. parvulus* 2.6 EPS is synthesized by the GTF glycosyltransferase encoded by the *gtf* gene, which is located in the pPP2 plasmid (Werning et al., 2006). Thus, the 2.6NR strain was generated from 2.6 by pPP2 plasmid curing after treatment with the DNA intercalating agent ethidium bromide and the gyrase inhibitor novobiocin (Fernández et al., 1995).

Consequently, given that 2.6NR does not utilize sorbitol, it was feasible that the *gut* operon was encoded by pPP2 and this hypothesis was investigated. First, total plasmidic DNA preparations of the two *Pediococcus* strains were purified by fractionation in a CsCl gradient to eliminate non-supercoiled (open circles and linear) forms of the plasmids. Then, the purified plasmidic DNA preparations were analyzed in an agarose gel (**Figure 4**). Four and three bands were detected, respectively, in preparations of the 2.6 and 2.6NR strains. The sizes of the bands were inferred from their migration using a calibration curve (**Figure 4B**) generated with the plasmids of the *E. coli* V517 strain and are shown in **Figure 4A**. Two of the bands apparently were shared by 2.6 and 2.6NR, and were initially ascribed to the monomeric forms of pPP1 (39.1 kpb in 2.6 and 40.0 kpb in 2.6NR) and pPP3 (12.7 kpb). As expected, pPP2 (24.5 kpb) was not detected in 2.6NR DNA preparations. Moreover, we could not ascribe to any plasmid the band with less mobility and a

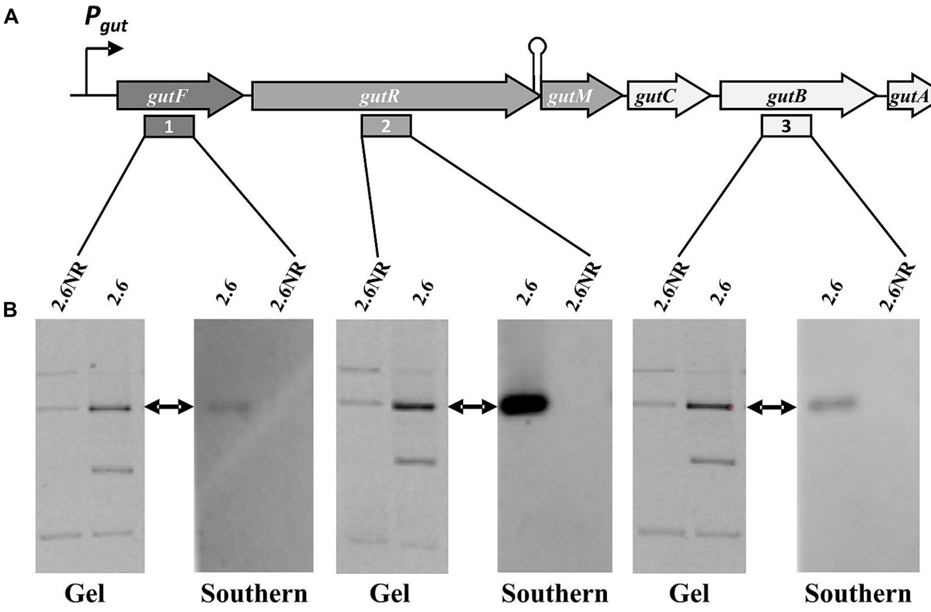


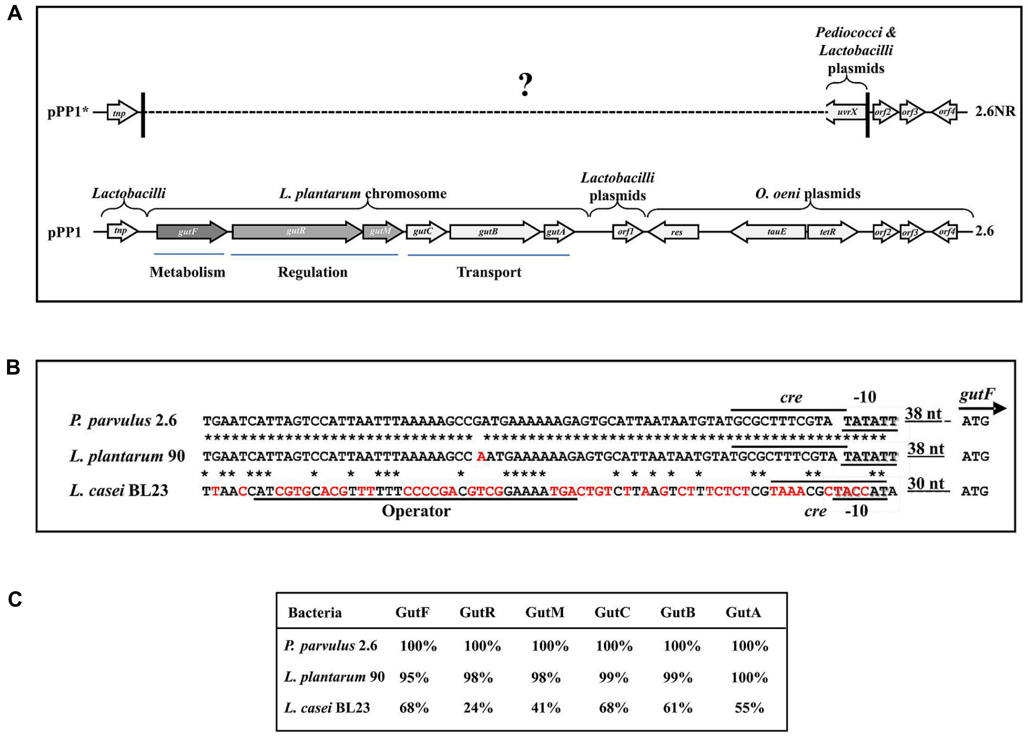
FIGURE 5 | Detection of the *P. parvulus* 2.6 *gut* operon by Southern blot hybridization. Plasmids preparations of the *P. parvulus* strains were fractionated by electrophoresis in agarose gel, transferred to membranes and hybridized for detection of *gutF*, *gutR* and *gutB* with the probes 1, 2, and 3, respectively. **(A)** Physical map of the *gut* operon, including the putative promoter of the *gut* operon (P_{gut}), the operon genes (*gutF, gutR, gutM gutC, gutB* and *gutA*), a secondary structure including the 3′-end of *gutR* and the 5′-end of *gutM* (coordinates 3483-3512 in GenBank accession No MF766019) as well as a transcriptional terminator (coordinates 6103-6027 in GenBank accession No MF766019) downstream of *gutA.* In addition, location of probes 1, 2, and 3 is indicated. **(B)** Images of the corresponding gels and hybridized membranes. The double headed arrows indicate the position of the hybridized bands in the corresponding gel.

theoretical molecular weight of 56.8 kbp that was present in DNA preparations of both strains. Quantification of the bands from agarose gels (**Figures 5**, **6**) revealed different proportions of the plasmidic forms in 2.6 (0.3:5.9:2.5:1.0) and 2.6NR (0.8:1.0:0.0:1.0) samples.

The *gut* operon of *P. parvulus* 2.6 (**Figures 5A**, **6A**) is composed of six genes, of which *gutF* encodes a sorbitol-6-phosphate dehydrogenase; *gutRM* encodes two putative regulators; and *gutCBA* encodes the proteins EIIC, EIIBC and EIIA which are components of a phosphoenolpyruvate-dependent sorbitol phosphotransferase system (PTS^{gut}). Thus, to detect the location of the *gut* operon, Southern blot hybridization of total plasmidic DNA preparations was performed using as a probe internal regions of *gutF*, *gutR* or *gutB*. One hybridization signal was observed with the three probes at the position of the 39.1 kb pPP1 plasmid in the 2.6 DNA sample (**Figure 5B**). Surprisingly, this plasmid was apparently present in both *P. parvulus* strains, but in the 2.6NR DNA sample no signal was observed. Nevertheless, the results demonstrated that the *gut* operon was not located in the pPP2 plasmid, but rather was carried by the pPP1 plasmid of the 2.6 strain and not of the newly designated pPP1* plasmid of 2.6NR strain.

Analysis of Plasmids pPP1 of *P. parvulus* 2.6 and pPP1* of *P. parvulus* 2.6NR

The results obtained by Southern blot analysis prompted us to obtain further information of pPP1 and pPP1* plasmids. Thus, the total plasmidic DNA preparation of the 2.6 strain was used as a substrate to confirm the sequence of the gut operon and to determine the unknown nucleotide sequence of the flanking regions (undetected in the draft genome of the bacterium) by the dideoxynucleotide method and with the walking strategy. The sequence of a DNA segment of 11,746 bp (**Figure 6A** and GenBank accession No MF766019) was obtained and its analysis revealed the existence of nine open reading frames (ORF), in addition to the 6 genes (*gutFRMCBA*) of the *gut* operon (**Figure 6A** and Supplementary Table S1). One open reading frame was detected upstream of the gut operon and was designated *tnp*, since its product has 100% identity with a multispecies transposase (Genbank accession No WP_003606336.1) widely distributed in the Lactobacillaceae family. Downstream of the gut operon were detected four ORF named orf1, orf2, orf3 and orf4, which could encode hypothetical proteins conserved in other LAB. In addition, the product of the named *res* gene belongs to the Ser-recombinase superfamily (cl02788) and specifically to the PinE conserved protein domain family (COG1961), showing more than 90% amino acid identity with proteins from oenococci, lactobacilli and pediococci annotated as Pin-related site-specific recombinases/DNA invertases. Also, two divergent genes named *tauE* and *tetR* seem to encode a TauE sulfite exporter which belongs to the TauE conserved domain family (pfam01925) and a transcriptional regulator belonging to the TetR family (domain architecture ID 11442015), and both proteins have more than 95% amino acid identity with their homologues in *Oenoccocus oeni* and lactobacilli.



Bacteria	GutF	GutR	GutM	GutC	GutB	GutA
P. parvulus 2.6	100%	100%	100%	100%	100%	100%
L. plantarum 90	95%	98%	98%	99%	99%	100%
L. casei BL23	68%	24%	41%	68%	61%	55%

FIGURE 6 | The sorbitol operon and flanking DNA regions of *P. parvulus* 2.6. **(A)** Sequenced regions of pPP1 and pPP1* plasmids are shown. Genes and ORF are depicted and homologies at the nucleotide level are indicated (see details in the text). ? Indicates unknown sequence. Homology of regulatory genetic regions **(B)** and proteins **(C)** involved in sorbitol utilization between *P. parvulus* 2.6, *L. plantarum* 90 and *L. casei* BL23 is shown. **(B)** DNA sequences of: the putative P_{gut} promoters (–10 boxes) and the GutR operator of *L. casei* are underlined; the putative *cre* sequences for catabolic repression are overlined; also the start codon (ATG) of *gutF* gene and the nucleotides between this and the –10 boxes are depicted. Red letters indicate mismatches with the *P. parvulus* 2.6 DNA sequence. *Means identity of nucleotides. **(C)** Identity of the amino acid sequence of the Gut proteins from *L. plantarum* 90 and *L. casei* BL23 with those of *P. parvulus* 2.6 is depicted.

Based on the DNA sequence of the *gut* operon flanking regions in pPP1, and on the lack of the *gut* operon in 2.6NR, primers were designed and used to try to detect if there exists any identity between pPP1 and pPP1* by DNA sequencing. Two of these, pPP1*F and pPP1*R, located respectively upstream and downstream of the *gut* operon, provided the desired information (**Figure 6A**). A good chromatogram of the DNA sequencing of pPP1* using the 2.6NR plasmidic preparation and pPP1*F primer with 100% identity with pPP1 was obtained until nucleotide 156 in the chromatogram (548 nt in Genbank accession No WP_003606336.1), then at least two overlapping sequences were observed (Supplementary Figure S2A), and it was not possible from this point to deduce a further correct DNA sequence. This was not the case when DNA from the 2.6 strain was used as substrate, since a good chromatogram of the pPP1 DNA sequencing was obtained (Supplementary Figure S2B). However, the usage of pPP1*R allowed not only to determine that the homology between pPP1 and pPP1* starts again at nucleotide 10,021 (in Genbank accession No WP_003606336.1), but also that upstream of this position in pPP1* there exists a region including a *uvrX* putative gene identical to those of other pediococci (i.e., in pPC892-2 plasmid, Genbank accession No CP021472.1) and *Lactobacilli* (i.e., in pH10 plasmid, Genbank accession No CP002430.1) plasmids, which do not carry *orf2, orf3 and orf4*.

With regard to the *gut* operon of *P. parvulus* 2.6, the identity of the region including the genes and the upstream regulatory regions with the homologues of *L. plantarum* strains was 99% (**Figures 6A,B**) and nucleotides from 694-to 60020 in GenBank accession No MF766019), consequently the amino acid sequence of the Gut proteins of *P. parvulus* showed an identity ranging from 95 to 100%, with those of *L. plantarum* 90 (**Figure 6C**). No significant homology at the DNA sequence level was detected between the characterized operons of *L. casei* and those of *P. parvulus* (**Figure 6B** and results not shown). However, presumably due to convergent evolution, homology ranging from 68 to 24% amino acid identity was detected between the Gut proteins of *P. parvulus* 2.6 and of *L. casei* BL23 (**Figure 6C**).

Analysis of the *Gut* Operon Regulation

GutR and GutM of *P. parvulus* could be involved in regulation of the *gut* operon expression and upstream of the start codon of *P. parvulus* 2.6 *gutF* gene, a TATAtT sequence was detected that only deviates one nucleotide from the consensus −10 promoter region (**Figure 6B**). Thus, to gain insight into this potential regulation, complementation studies in heterologous LAB hosts able to utilize sorbitol were carried out. First, we cloned

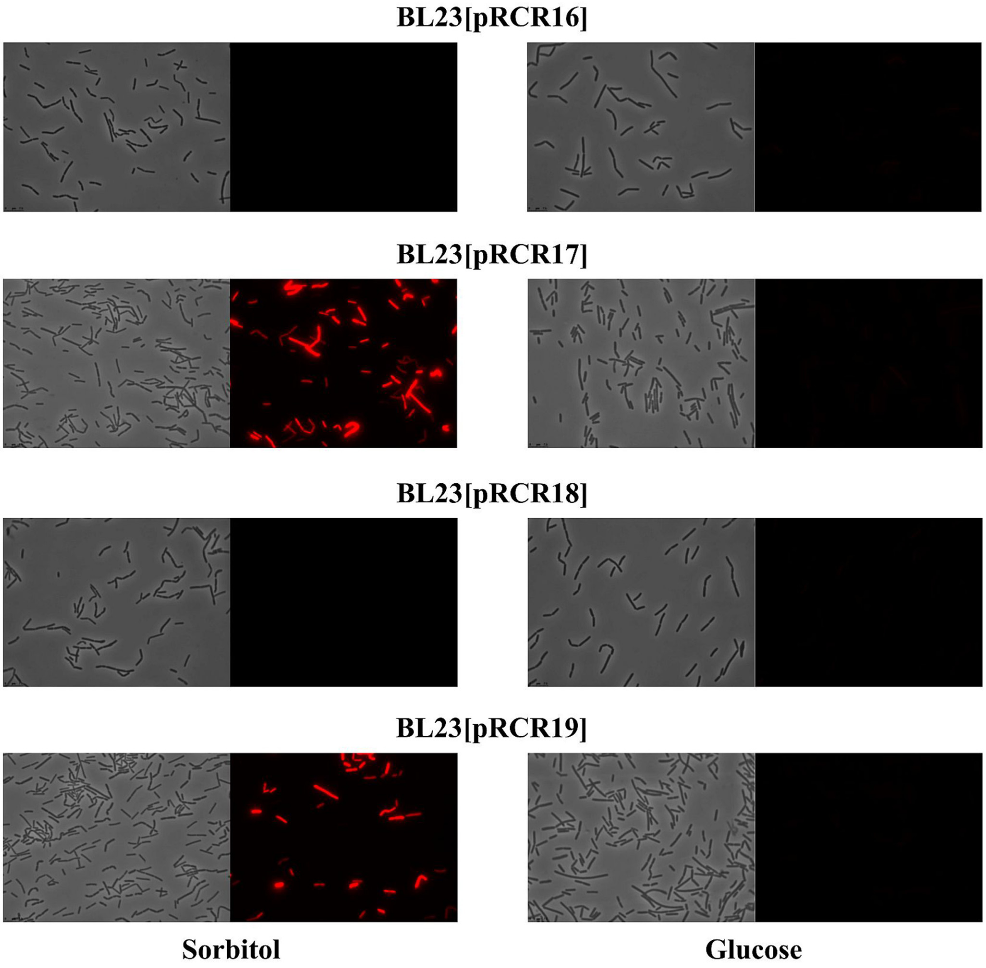


FIGURE 7 | Detection of fluorescence in *L. casei* BL23 strains at stationary phase. Cultures of the indicated strains in MRSS (sorbitol) or MRSG (glucose) were analyzed by phase contrast (left panels) or fluorescence (right panels) microscopy.

independently the putative promoter sequence (designated P_{gut}) and its upstream region (**Figure 6B**), as well as the transcriptional fusions P_{gut}-*gutR*, P_{gut}-*gutM* and P_{gut}-*gutRM* into the pRCR promoter probe vector (Mohedano et al., 2015) upstream of the *mrfp*, generating the pRCR16, pRCR17, pRCR18 and pRCR19 plasmids, respectively (**Figure 1**). Thus, functionality of the promoter and influence of GutR and GutM could be detected by measuring the levels of fluorescence of the mCherry encoded by the *mrfp* gene. As hosts to perform the studies, we chose: (i) the plasmid free *L. casei* BL23, because its sorbitol utilization and the regulation of its *gut* operon is known (Yebra and Pérez-Martínez, 2002; Nissen et al., 2005; Alcantara et al., 2008) and, (ii) *L. plantarum* 90, because we have previously detected in this bacterium efficient functional expression of mCherry from a pRCR derivative, without problems of plasmid incompatibility and that the copy number of the plasmid was 62 ± 2 molecules per bacterial genome (Russo et al., 2015).

The well characterized transcriptional activator GutR of *L. casei* BL23 controls expression of the *gut* operon of this bacteria and its operator site upstream of the P_{gut} has been identified as well as a catabolite repression element (*cre*) overlapping the −10 region of the promoter (Alcantara et al., 2008) (**Figure 6B**). The *P. parvulus* 2.6 GutR has only a low homology of amino acids (24%) with its homologue of *L. casei*, but like its counterpart belongs to the BglG transcriptional antiterminators family, possesses the PRD domain and the DNA helix turn helix binding domain. Therefore, both proteins could have a similar role. Alignment of the *L. casei* and *P. parvulus* -10 regions revealed that the upstream regulatory regions of BL23 strain has no clear homologs in the 2.6 strain (**Figure 6B**). Consequently, cross talk between transcriptional signals of *P. parvulus* and *L. casei* regulators should not take place, and influence of the pediococcal GutR and GutM in expression of P_{gut} from the 2.6 strain could be investigated in the BL23 strain without interferences. Thus, the pRCR derivatives were transferred independently to the BL23 strain and the recombinant bacteria were grown in MRS supplemented with either 1% glucose or 1% sorbitol until stationary phase prior to analysis. Examination of the cultures by fluorescent and phase contrast optical microscopy revealed that only bacteria carrying pRCR17 and pRCR19 and grown in

TABLE 3 | Heterologous expression of components of the *P. parvulus* 2.6 *gut* operon in *L. casei* BL23 carrying pRCR derivatives plasmids grown in either MRSS or MRSG.

***L. casei* strains**	**DNA insert in pRCR derivatives**	**Specific fluorescence[a]**	
		MRSS	**MRSG**
BL23[pRCR16]	P_{gut}	0.14 ± 0.07	0.14 ± 0.01
BL23[pRCR17]	P_{gut}-*gutR*	5.69 ± 0.44	0.07 ± 0.09
BL23[pRCR18]	P_{gut}-*gutM*	0.19 ± 0.06	0.11 ± 0.02
BL23[pRCR19]	P_{gut}-*gutRM*	3.58 ± 0.06	0.05 ± 0.02

[a] *The specific fluorescence is depicted and it was calculated as the ratio of the detected fluorescence (5×) and the bacterial biomass estimated from the* $OD_{600\ nm}$ *of the culture.*

presence of sorbitol have fluorescence (**Figure 7**). In addition, fluorescence as well as the optical density of the cultures was measured and the specific fluorescence, referred to the biomass, was calculated. The fluorescence quantification confirmed that the P_{gut}-*gutRmrfp*, and P_{gut}-*gutRMmrfp* transcriptional fusions are activated upon growth in the presence of sorbitol (**Table 3**). Thus, these results revealed that expression from the P_{gut} required the activation by GutR and the presence of sorbitol in the growth medium. Moreover, they indicated that activation by GutR decreased, when GutM was present (5.69 ± 0.44 *versus* 3.58 ± 0.06).

Concerning the *L. plantarum* 90 host, its GutR has 98% homology to that of *P. parvulus* 2.6 (**Figure 6C**) and the DNA sequence of the region located upstream of the two P_{gut} promoters only differs in one nucleotide (**Figure 6B**). Consequently, both operons must have the same regulatory gene system, which implies that both systems could recognize each other. Thus, a trans-complementation process was expected between the regulatory proteins of Lp90 and the promoter region of 2.6. Therefore, the pRCR derivatives were transferred independently to the 90 strain and, since a cross talk is more complex situation, its comprehension required a more detailed analysis. For this reason, the recombinant bacteria, after growth in MRS supplemented with 1% glucose, were transferred to MRS fresh medium supplemented with either 1% sorbitol or 1% glucose and a time course assay of fluorescence and growth of the cultures was performed. The results revealed that all recombinant strains became fluorescent, when grown in the presence of sorbitol and, with the time of incubation the fluorescence increased (**Figure 8** and **Table 4**). In addition, analysis of the bacterial growth showed that all cultures in MRSG have very similar exponential growth rates (ranging from 0.889 ± 0.059 to 0.803 ± 0.049) and all entered slowly into stationary phase after 2 h of incubation (**Figure 8F** and **Table 4**). Initial transfer of the cultures to MRSS resulted in a similar decrease (around 50%) of the growth rate (values from 0.416 ± 0.045 to 0.495 ± 0.011) during the first 2 h of induction. Then, probably after consumption of the residual intracellular glucose or due to the induction process, bacteria decreased their growth rate to levels ranging from 0.259 ± 0.020 to 0.251 ± 0.034, besides the 90[pRCR18] (GutM overexpressor), that after stalling its growth from 2 h to 3 h incubation time decreased its growth rate to 0.239 ± 0.048, indicating that overexpression of GutM in absence of high levels of GutR has a negative impact for the cells. Furthermore, analysis of the specific levels of fluorescence of the cultures referred to their biomass (**Table 4**) showed different levels for the different fusions (P_{gut}-*mrfp* < P_{gut}-*gutRMmrfp* < P_{gut}-*gutRmrfp* < P_{gut}-*gutMmrfp*), showing that overexpression of GutM provokes the highest induction of expression from P_{gut}. In addition, the highest levels were observed after 4 h of induction for cells carrying either pRCR17 (22.38 ± 2.02) or pRCR19 (19.26 ± 2.10) *versus* the end of the incubation (6 h) for cells carrying pRCR16 (14.81 ± 0.66) and pRCR18 (26.83 ± 1.83).

Thus, the results revealed a trans-complementation of the *L. plantarum* regulatory proteins on expression driven from the *P. parvulus* P_{gut} promoter. Moreover, the results confirmed the role of inducer of GutR as well as requirement of sorbitol for expression from P_{gut} and support that co-expression of GutR and GutM decrease the activation mediated by GutR.

DISCUSSION

The overall metabolic results obtained here support that *P. parvulus* is able to synthesize EPS in MRS medium using either glucose or sorbitol as carbon sources. We have previously demonstrated that the 2-substituted (1,3)-β-D-glucan of *P. parvulus* 2.6 is synthesized by the GTF glycosyltransferase utilizing UDP-glucose as substrate (Werning et al., 2014). In addition, Velasco et al. (2007) determined that the 2.6 strain transport the glucose by a PMF-permease and possesses the α-phosphoglucomutase and the UDP-glucose pyrophosphorylase activities responsible for the conversion of glucose-6-P to glucose-1-P and further conversion of this compound to UDP-glucose. Thus, Velasco et al. (2007) showed how the 2.6 strain uses the glucose, not only for the central metabolism, *via* the glycolytic pathway, but also for the secondary metabolism involving a biosynthetic pathway for its EPS synthesis. In addition, the detection of the genetic determinants of sorbitol utilization by the 2.6 strain obtained in this work supports that the bacterium transports sorbitol by a PTS^{gut} system and converts sorbitol-6-P into fructose-6-P by the action of sorbitol-6-P dehydrogenase. Fructose-6-P can be converted to glucose-6-P by a reaction catalyzed by phosphoglucose isomerase, enzymatic activity that was also previously detected in the 2.6 strain (Velasco et al., 2007). Therefore, the 2.6 strain possesses the transport and enzymatic machineries for synthesis of the EPS from sorbitol. In addition, we have detected that aeration of the cultures during the growth improves sorbitol consumption (results not shown). Accordingly, the conversion of sorbitol-6-P into fructose-6-P requires NAD^+ as an oxidative co-factor to produce NADH (Zarour et al., 2017). Analysis of the draft genome of the 2.6 strain showed the existence of a putative NADH oxidase coding gene. If this enzyme exists, it could unbalance the NAD^+/NADH equilibrium toward the oxidized form NAD^+.

The *P. parvulus* 2.6 2-substituted (1,3)-β-D-glucan is composed of molecules of glucose and consequently the

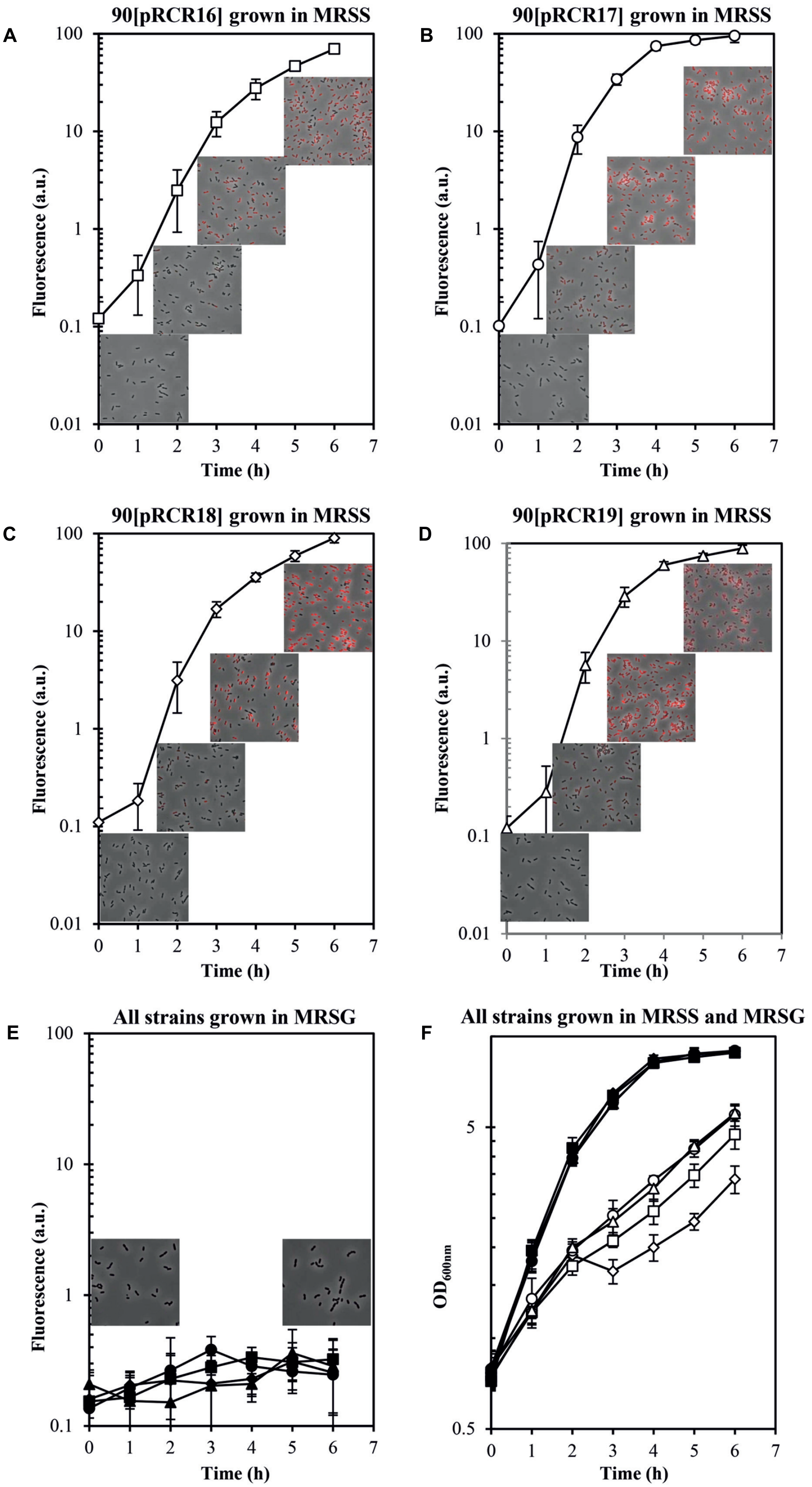


FIGURE 8 | Trans complementation of the sorbitol utilization regulatory machinery in *L. plantarum* 90. Bacteria carrying **(A,E,F)** pRCR16 (□, ■), **(B,E,F)** pRCR17 (○, ●), **(C,E,F)** pRCR18 (◇, ♦) or **(D,E,F)** pRCR19 (△, ▲) were grown in MRSG and at time 0 were transferred to fresh MRSS (open symbols) or to MRSG (closed symbols). OD_{600nm} and fluorescence of the cultures were monitored every hour. Overlays of images of the cultures taken at time 0 and after 2, 4, and 6 h incubation by phase contrast and fluorescent microscopy are depicted. The experiments were performed in triplicate and the mean value and standard deviation is depicted.

TABLE 4 | Heterologous expression of components of *P. parvulus* 2.6 *gut* operon in *L. plantarum* 90 carrying pCRC derivatives plasmids.

L. plantarum strains	DNA insert in pRCR derivatives	Medium	Initial growth rate[a] (μ)	Sorbitol induced growth rate[b] (μ)	Specific fluorescence[c]				
					2 h	3 h	4 h	5 h	6 h
90[pRCR16]	P_{gut}	MRSS	0.416 ± 0.045	0.251 ± 0.034	1.48 ± 1.03	5.86 ± 1.43	10.42 ± 1.56	13.44 ± 0.51	14.81 ± 0.66
90[pRCR17]	P_{gut}-*gutR*	MRSS	0.455 ± 0.065	0.259 ± 0.020	4.42 ± 1.22	13.41 ± 0.90	22.38 ± 2.02	20.30 ± 1.62	17.28 ± 1.65
90[pRCR18]	P_{gut}-*gutM*	MRSS	0.429 ± 0.025	0.239 ± 0.048	1.62 ± 0.69	10.26 ± 2.30	18.05 ± 2.85	24.26 ± 1.51	26.83 ± 1.83
90[pRCR19]	P_{gut}-*gutRM*	MRSS	0.495 ± 0.011	0.256 ± 0.016	2.80 ± 0.77	11.74 ± 1.51	19.26 ± 2.10	17.20 ± 1.71	15.98 ± 1.21
90[pRCR16]	P_{gut}	MRSG	0.889 ± 0.059	*ND*	0.06 ± 0.03	0.04 ± 0.00	0.04 ± 0.00	0.04 ± 0.01	0.04 ± 0.01
90[pRCR17]	P_{gut}-*gutR*	MRSG	0.825 ± 0.029	*ND*	0.07 ± 0.06	0.06 ± 0.02	0.04 ± 0.01	0.03 ± 0.00	0.03 ± 0.02
90[pRCR18]	P_{gut}-*gutM*	MRSG	0.803 ± 0.049	*ND*	0.06 ± 0.03	0.03 ± 0.03	0.03 ± 0.01	0.04 ± 0.01	0.03 ± 0.01
90[pRCR19]	P_{gut}-*gutRM*	MRSG	0.832 ± 0.034	*ND*	0.04 ± 0.02	0.03 ± 0.02	0.03 ± 0.01	0.04 ± 0.02	0.03 ± 0.02

[a]Growth rate of the cultures grown in MRSS was calculated from the $OD_{600\ nm}$ obtained during the first 2 h of induction. [b]Growth rate of the cultures grown in MRSS was calculated from the $OD_{600\ nm}$ obtained during the 2–6 h of induction, besides for 90[pRCR18], that due to the stalling of growth from 2 h to 3 h of incubation the growth rate was calculated from the data obtained from 3 h to 6 h of incubation. ND, the growth rate was not determined because the cultures have entered in the stationary phase of growth. [c]Specific fluorescence was calculated as the ratio of the detected fluorescence (5x) and the bacterial biomass estimated from the OD_{600} nm of the culture.

EPS concentration can be calculated as molarity of this monosaccharide (see secondary Y axis in **Figure 3A**). This calculation revealed that in both media this bacterium only used a small percentage of the substrate molecules (10 mM glucose plus 30 mM sorbitol in MRSGS or 10 mM glucose in MRSG) for synthesis of EPS (0.99 or 0.45 mM, respectively), whereas more than 90% was utilized in the glycolytic pathway to synthesize pyruvic acid (2 molecules per 1 molecule of substrate) and by action of the lactate dehydrogenase to finally generate lactic acid (1 molecule per 1 molecule of pyruvate, 79 mM or 18 mM). Moreover, the specific quantification method for 2-substituted (1,3)-β-D-glucan used here and the estimation of the specific concentration of EPS synthesized (**Figure 3C**) showed that, using as substrate either glucose or sorbitol, the bacterium synthesizes the same polymer and suggests that with the same efficiency. This was not the case when synthesis of this EPS utilizing fructose was tested, since levels were low compared with that obtained from glucose (Velasco et al., 2007). We have also observed a temporal delay of 2.6 to start to consume sorbitol in MRSGS (**Figure 3A**). This could be due to the existence of a catabolite repression of sorbitol utilization by glucose. Supporting this hypothesis, we have detected a potential *cre* operator (**Figure 6**) for the CcpA, which mediates with HPR this regulation in firmicutes (Deutscher, 2008).

In *P. parvulus* 2.6, the *gut* operon, as in other LAB, constitutes the genetic determinant for sorbitol transport and conversion into fructose-6-P. In addition, we have established here that it is located in a plasmid named pPP1 (**Figure 6**) which is unusual, since the almost identical operon of *L. plantarum* and that of *Lactobacillus pentosus* strain SLC13 (82% homologous, Genbank accession No CP022130.1) as well as the unrelated one from *L. casei* are located in the chromosome. As far as we know, only the location of an unrelated *gut* operon in the megaplasmid pMP118 from *L. salivarius* UCC118 has been previously described (Claesson et al., 2006). A search of the protein data banks revealed that *L. salivarius* 5713 and JCM1046 strains possess, respectively, the pHN3 and pMP1046A megaplasmids which carry *gut* operons homologous to that of pMP118 (Jiménez et al., 2010; Raftis et al., 2014). Flanking the operon two inverted repeat sequences (nucleotides 604-627 and 6612-6635 of Genbank accession No WP_003606336.1) were identified, which are also present at the same relative location in *L. plantarum* strains and at various locations in lactobacilli chromosomes and plasmids (even more than one copy per genome). The upstream region is preceded by a *tnp* gene encoding a putative transposase, which could be responsible for mobilization of the *gut* operon from plasmid to chromosome or *vice versa*.

Lactic acid bacteria are prone to carry more than one compatible plasmid and this facilitates exchange of different regions with physiological significance, that later on can be transferred to other bacteria by plasmid conjugation or mobilization (Cui et al., 2015). Thus, downstream of the *gut* operon of pPP1 there are DNA regions almost identical to that present in plasmids of lactobacilli, which along with *P. parvulus* can be contaminants of alcoholic beverages. Furthermore, the

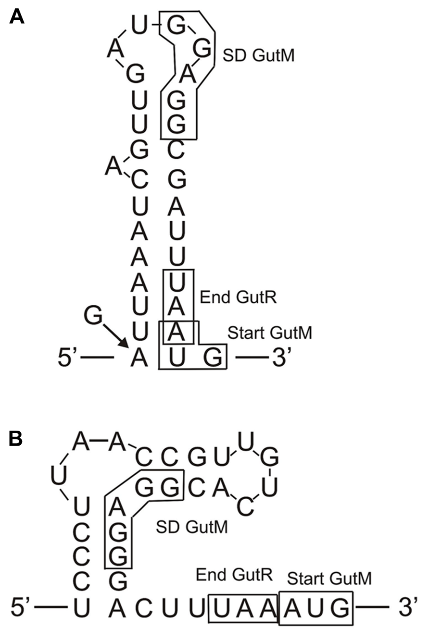


FIGURE 9 | Predicted folding of the *gut* mRNA region including the 3′-end of *gutR* and the 5′-end of *gutM*. **(A)** Secondary structure of the wild-type *P. parvulus* 2.6 and *L. plantarum* 90 *gut* operons. The arrow and the G indicate that in the insert of pRCR18 G substitute to the A in the indicated position. **(B)** Secondary structure of the *L. casei* BL23 *gut* operon.

Oenococcus oeni pOENI-1 and pOENI-1v2 plasmids (Favier et al., 2012) and pPP1 carry a region containing among others the *res, tauE* and *tetR* genes. The putative TauE sulfite exporter is possibly involved in adaptation to stress conditions during alcoholic beverage production (Favier et al., 2012). Thus, the recombinase or invertase site specific Res could be responsible for a mobilization of an element composed of a truncated *res, tauE* and *tetR* to a stable location, since at the 3′-end region of *res* and downstream of *tetR* unit exist inverted complementary sequences 5′-TTTTAAAGC-3′ and 5′-GCTTTAAAA-3′ (nucleotides 7774–7778 and 10021–10029 of Genbank accession No WP_003606336.1).

Another instance of plasmids rearrangement in *P. parvulus* is that which generated the profile and DNA sequence of 2.6NR strain plasmids (**Figure 6**). The initial isolate of 2.6NR strain generated in the Basque country University (BCU, Spain) and described in Fernández et al. (1995), kindly provided by Dr. Maria Teresa Dueñas (BCU) was studied in this work. Thus, the changes in plasmid cassettes were not produced in our laboratory, and presumably they took place upon treatment of 2.6 strain with ethidium bromide and novobiocin and was selected for the loss of the ropy phenotype. Thus, it is feasible that a formation of a co-integrate of pPP1 with other plasmid, may be pPP2, took place and convergent replication from two origins prompted to a deletion of one of the replicons, may be the pPP2, since its loss was envisaged, to generate pPP1*.

Concerning the regulation of expression of the *gut* operon, the overall results showed that it is repressed in the absence of sorbitol in the growth medium and that the *P. parvulus* GutR is an activator like the *L. casei* BL23 regulator (Alcantara et al., 2008). In this system, it has been proposed that GutM is involved in the activation, since a decreased expression of the *gut* operon was detected in a GutM deficient mutant (Alcantara et al., 2008). Furthermore, the complementation studies in *L. plantarum* 90 performed here showed a heterologous regulation of gene expression from the pediococcal P_{gut} promoter by the GutR from *Lactobacillus*, and a positive effect when only the pediococcal GutM was overexpressed (**Figure 8** and **Table 4**). Thus, these results suggest that a protein-protein interaction between the *P. parvulus* GutM and the *L. plantarum* GutR could potentiate the activation of the P_{gut} promoter, since, *P. parvulus* 2.6 GutR and GutM have 98% identity with their homologues of *L. plantarum* 90. In addition, either in *L. casei* BL23 and in *L. plantarum* a decrease of expression from P_{gut} was observed when GutM was overexpressed in combination with GutR (**Figure 8** and **Tables 3**, **4**). This prompted us to analyze the genetic environment of *gutR* and *gutM.* An overlapping of the last nucleotide of the termination codon (TAA) of *gutR* and the first nucleotide of the start codon (ATG) of *gutM* was detected in *P. parvulus* 2.6 and *L. plantarum* 90 genomes. This indicated that post-transcriptional regulation of the *gut* operon could exist in this bacterium. For this reason, the secondary structure of the region surrounding the overlapping in the *gut* mRNA was folded with the Mfol program (**Figure 9A**). A secondary stem-loop structure was predicted with a $\Delta G = -5.6$ kcal mol^{-1}, the ribosomal binding site (RBS) of *gutM* (5′-GGAGG-3′) was located at the loop and partially blocked in the stem of the structure. Thus, even though the sequence of the RBS of *gutM* indicates a high efficiency of utilization for the ribosome, the initiation of translation of *gutM* could be partially impaired by the partial RBS blockage, which would be released by the opening provoked by the passage of the ribosomes translating *gutR*. In addition, the overlapping of *gutR* and *gutM* is located at the end of the stem of the structure. Thus, two post-transcriptional regulations could take place: (i) translation of *gutR* can act by favoring translation of *gutM* by exposition of its RBS and (ii) a -1 frameshift (Atkins et al., 2016) could happen at the TAA termination codon of *gutR* and ribosomes translating this could step back one nucleotide and upon charging the corresponding tRNA read the Leu (TTA) codon and continue translating *gutM.* In this way a fused peptide GutR-M could be synthesized. The same structure could be formed in the transcript encoded by the plasmid pRCR19 with a $\Delta G = -5.9$ kcal mol^{-1}, containing *gutRM* and which could be a substrate for the two proposed post-transcriptional regulations. Furthermore, the DNA fragment cloned in pRCR18, lacks most of the *gutR* gene but still retains some of the 3′-end region of this gene and the encoded mRNA can form a secondary structure almost identical to the wild-type structure (with only a change of A-U by G-U pairing at the end of the stem, **Figure 9**). Thus in bacteria carrying pRCR18 partial blockage of the RBS could take place, but synthesis of GutR-M could not occur. This could explain the antagonistic effect of overexpression of *gutM* from pRCR18 (increase of expression from P_{gut}) and pRCR19 (decrease of expression from P_{gut}), if GutR-M exists and has a role.

Prediction of transmembrane regions in the regulatory proteins with the TM-Pred revealed that GutR is a soluble protein and that the first amino acids from 1 to 21 of GutM constituted a transmembrane region also predicted for the GutR-M fused polypeptide. This fused polypeptide could provide an efficient anchoring of the regulator to the membrane bringing it close to the PTS^{gut} system facilitating the phosphorylation of GutR and resulting in the physiological optimal expression of the operon. This generation of a fused polypeptide could also take place in *L. plantarum* but does not seems to occur in *L. casei,* since in this bacterium the TAA translational stop codon of GutR and the ATG start codon of GutM are adjacent and not overlapped (**Figure 9**). However, the *L. casei gut* transcript can form a secondary structure with a $\Delta G = -9.8$ kcal mol^{-1} which could block the RBS of *gutM* gene, couple translation of GutR and GutM could take place, and protein-protein interaction could be responsible for higher activation of the system at the beginning of the induction process. Our results indicate that high levels of GutM synthesized from a multicopy plasmid have a deleterious effect for the bacteria (**Figure 8**) and probably the proposed models of posttranscriptional regulation are designed to have the right concentration of regulatory proteins. Nevertheless, further experiments are required to pinpoint the role of GutM and of the putative GutR-M polypeptide of *P. parvulus*.

AUTHOR CONTRIBUTIONS

AP-R contributed to all parts of the experimental work and wrote a draft of the manuscript. MW performed the initial detection of sorbitol utilization and characterization of *gut* genes. AP contributed to the characterization of the sorbitol metabolism. PR participated in the elaboration of the manuscript and analysis of the DNA sequences. GS contributed to the design and analysis of the experimental work involving characterization of regulation of *gut* operon expression. MM contributed to the design of strategies to determine trans complementation of the *gut* operon and corrected the manuscript. PL participated in study conception, data interpretation and generated the final version of the manuscript. All authors have read and approved the final manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2017.02393/full#supplementary-material

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β-glucan-producing *Pediococcus parvulus* 2.6: test of probiotic and immunomodulatory properties in zebrafish models. (2018) *Frontiers in* Microbiology 9, 1684

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CHAPTER 5

Resumen

En el sector de la acuicultura, los β-glucanos de origen fúngico son ampliamente utilizados para la prevención de brotes infectivos de origen bacteriano y vírico. Sin embargo, el uso de antibióticos sigue siendo la práctica más habitual, provocando la aparición de cepas resistentes y debilitando tanto la microbiota como el sistema inmune de estos animales.

Muchas bacterias del ácido láctico (BAL) forman parte de la microbiota de humanos y otros animales, ejerciendo importantes beneficios para su salud y por este y otros motivos, a muchas de estas bacterias se las consideran organismos probióticos. Así, a nivel del sistema digestivo contribuyen a reestablecer y mantener la homeostasis microbiana, a fortalecer de la barrera intestinal, a combatir a los patógenos y a estimular el sistema inmune. Además, ciertos biopolímeros como los EPS producidos por BAL, son considerados como importantes agentes inmunoestimulantes. De este modo, la implementación del uso de probióticos que a su vez produzcan EPS inmunoestimulantes puede ser una práctica muy beneficiosa en este campo. Por este motivo, en este trabajo se han estudiado las propiedades probióticas de la bacteria *P. parvulus* 2.6, así como las propiedades inmunoestimulantes de su (1,3)-β-D-glucano ramificado en posición *O*-2 (β-glucano). Para ello se utilizaron modelos embrionarios de *Danio rerio* (pez cebra). Este modelo ha emergido, por sus numerosas ventajas frente a los modelos *in vivo* tradicionales, como una interesante alternativa para la evaluación de las propiedades probióticas de bacterias. Así, en trabajos previos nuestro grupo ya ha utilizado este modelo para la evaluación de BAL de las especies *Lactobacillus fermentum*, *Lactobacillus plantarum*, *Lactobacillus sakei* y *Leuconostoc mesenteroides* (Russo et al., 2015; Nácher-Vázquez et al., 2017a; Zarour et al., 2018).

En este trabajo, *P. parvulus* 2.6 y su cepa isogénica 2.6NR no productora de β-glucano fueron marcadas fluorescentemente mediante la transferencia del plásmido pRCR12, lo que permitió su detección *in vivo* en el modelo del pez cebra. El plásmido pRCR12 fue desarrollado en nuestro laboratorio a partir del vector pRCR (generado en el Capítulo 3 de esta tesis), mediante la clonación del promotor neumocócico P_x (Russo et al., 2015). En las cepas de *P. parvulus* la presencia de pRCR12 conllevó a la expresión constitutiva de la proteína fluorescente mCherry, emitiendo altos niveles de fluorescencia y otorgando una coloración rosada intensa a sus colonias. Además, la presencia del

plásmido no afectó el crecimiento bacteriano. En este trabajo, también se valoró mediante el uso de un modelo *in vitro* la capacidad de adhesión de las bacterias recombinantes a la línea celular humana Caco-2. En un trabajo previo realizado con las estirpes parentales, ya se mostró que la cepa 2.6 presenta una mayor capacidad de adhesión a los enterocitos, que la cepa 2.6NR (Fernández de Palencia et al., 2009). En este trabajo, hemos demostrado que la presencia del plásmido pRCR12 no afecta a la capacidad de adhesión de las bacterias. La cepa 2.6[pRCR12] mostró mayor adhesión que 2.6NR[pRCR12] a las células eucarióticas intestinales. Para valorar la influencia del β-glucano en la adhesión, se cuantificó la cantidad de biopolimero que está presente durante los ensayos de adhesión utilizando el método inmunológico desarrollado en el Capítulo 1 de esta tesis, y se añadió esa cantidad a los cultivos de la cepa 2.6NR. De este modo, la unión a las células Caco-2 de la cepa 2.6NR[pRCR12] aumentó significativamente al realizarse el ensayo de adhesión en presencia del β-glucano purificado. De igual modo, al eliminar el EPS de los cultivos de 2.6, esta vio disminuida su capacidad de adhesión. Estos resultados confirmaron que el β-glucano influye favorablemente en la capacidad de adhesión de *P. parvulus*.

También, en este trabajo se han utilizado dos modelos embrionarios de pez cebra: (i) un modelo gnotobiótico para analizar la capacidad de colonización de las cepas recombinantes de *P. parvulus*, la competición de estas BAL frente a la infección producida por *Vibrio anguillarum* NB10[pOT11], y para la evaluación de la capacidad inmunomoduladora de su β-glucano; y (ii) un modelo donde la línea transgénica de pez cebra *Tg*(mpx:GFP) fue sometida a un proceso de inflamación inducida para analizar en ella la capacidad anti-inflamatoria del β-glucano.

Los resultados obtenidos mostraron como *P. parvulus* es capaz de colonizar el epitelio intestinal del pez cebra y como su presencia en el tracto digestivo disminuye con el trascurso del tiempo. Además, al igual que sucedía en el modelo *in vitro*, la presencia del β-glucano aumentaba la capacidad de colonización de la bacteria. Por otra parte, el pretratamiento de las larvas gnotobioticas con *P. parvulus* conllevó una disminución de la mortandad provocada en el pez cebra por la infección con el patógeno *V. anguillarum*. Este efecto fue superior cuando se utilizó la cepa 2.6[pRCR12] en lugar de 2.6NR[pRCR12], llegando a reducir en un 50% la mortalidad de las larvas provocada por la infección de *V. anguillarum* a las 72 horas de incubación.

Las propiedades inmunomoduladoras del β-glucano se analizaron primero con el modelo gnotobiótico, donde las larvas se sumergieron en soluciones del EPS a 150 µg mL^{-1} durante 30 horas. El análisis mediante RT-qPCR de la expresión génica de un conjunto de genes implicados en la respuesta inflamatoria, mostró que el β-glucano era capaz de reprimir la expresión de dos citoquinas pro-inflamatorias, IL8 y TNFα y de la proteína acopladora MyD88 (3,4 veces, 2,2 veces y 2,4 veces respectivamente).

Finalmente, se indujo un proceso inflamatorio en larvas de la línea *Tg*(mpx:GFP) mediante el seccionamiento de la región apical de la cola, y se analizó la migración y proliferación de los neutrófilos a través de la cuantificación de la fluorescencia debida a la proteína GFP expresada y emitida por estas células de las larvas. Los resultados que se obtuvieron revelaron que el β-glucano de *P. parvulus* tiene un efecto anti-inflamatorio, ya que las larvas tratadas con el β-glucano presentaban una disminución significativa en el reclutamiento de los neutrófilos en la región de inflamación, y su proliferación se vio inhibida.

Mi aportación a este trabajo

Para lograr la consecución de este trabajo científico, mi aportación fue la siguiente. Contribuí a la realización de todo el trabajo experimental. También, realicé el borrador del manuscrito, así como las figuras y tablas que contiene.

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β-Glucan-Producing *Pediococcus parvulus* 2.6: Test of Probiotic and Immunomodulatory Properties in Zebrafish Models

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Lactic acid bacteria synthesize exopolysaccharides (EPS), which could benefit the host's health as immunomodulators. Furthermore, EPS could protect bacteria against gastrointestinal stress, favoring gut colonization, thus protecting the host against pathogenic infections. *Pediococcus parvulus* 2.6, produces a 2-substituted (1,3)-β-D-glucan and, in this work, its probiotic properties as well as the immunomodulatory capability of its EPS have been investigated using *Danio rerio* (zebrafish). To this end and for a comparative analysis, *P. parvulus* 2.6 and its isogenic β-glucan-non-producing 2.6NR strain were fluorescently labeled by transfer of the pRCR12 plasmid, which encodes the mCherry protein. For the *in vivo* studies, there were used: (i) a gnotobiotic larvae zebrafish model for bacterial colonization, pathogen competition, and evaluation of the β-glucan immunomodulation capability and (ii) a transgenic (*mpx:GFP*) zebrafish model to determine the EPS influence in the recruitment of neutrophils under an induced inflammation. The results revealed a positive effect of the β-glucan on colonization of the zebrafish gut by *P. parvulus*, as well as in competition of the bacterium with the pathogen *Vibrio anguillarum* in this environment. The larvae treatment with the purified β-glucan resulted in a decrease of expression of genes encoding pro-inflammatory cytokines. Moreover, the β-glucan had an anti-inflammatory effect, when it was evaluated in an induced inflammation model of *Tg*(*mpx:GFP*) zebrafish. Therefore, *P. parvulus* 2.6 and its EPS showed positive health properties in *in vivo* fish models, supporting their potential usage in aquaculture.

Keywords: *Pediococcus parvulus*, exopolysaccharides, β-glucans, lactic acid bacteria, probiotics

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INTRODUCTION

Many lactic acid bacteria (LAB) reside in the intestinal tract of vertebrates. In addition, among these, it has been demonstrated that some strains belonging to the genera *Lactobacillus*, *Leuconostoc*, *Streptococcus*, and *Pediococcus* are probiotic, since they have beneficial effects for the health of the host organism (Guarner et al., 2017). The main mechanisms of action of probiotics are to strengthen the intestinal barrier, stimulate the immune system, or counteract the effects of pathogens through acidification of the environment, the production of antimicrobial substances, and competition for nutrients and adhesion to the epithelium (Guarner et al., 2017).

Many of these LAB produce a wide range of exopolysaccharides (EPS) that are considered prebiotic compounds (Salazar et al., 2016). These EPS could reach the colon where they are selectively fermented by the gut microbiota increasing beneficial anaerobic bacteria that contribute to the homeostasis and have beneficial effects on health (Verbeke et al., 2015). These bacteria and their EPS are used in the development of functional fermented foods (Zannini et al., 2016; Linares et al., 2017; Marco et al., 2017). Furthermore, the EPS, especially β-glucans, can act as immumodulators. β-Glucans are recognized by pattern recognition receptors such as dectin-1 and Toll-like receptors activating the NF-κB pathway that lead to the release of several cytokines (Chan et al., 2009; Lee and Kim, 2014). This activation stimulates the proliferation of monocytes and the maturation of dendritic cells and could be involved in anti-tumor responses (Meng et al., 2016).

Pediococcus parvulus 2.6 is a LAB isolated from Basque Country cider, whose genome has been determined (Pérez-Ramos et al., 2016). This bacterium produces a 2-substituted (1,3)-β-D-glucan of high molecular mass (Dueñas-Chasco et al., 1997). Evaluation of this bacterium using *in vitro* models showed the influence of β-glucan in some probiotic properties such as adhesion to human enterocyte cell lines and immunomodulation of macrophages (Fernández de Palencia et al., 2009). This β-glucan provided enhancement of bacterial growth and adhesion capability of lactobacilli (Russo et al., 2012), and was also able to activate human macrophages with an anti-inflammatory response (Notararigo et al., 2014). Furthermore, a *P. parvulus* 2.6 fermented cereal-based matrix, producing the β-glucan *in situ* resulted in improved technological and functional features of the products (Pérez-Ramos et al., 2017a). Moreover, these β-glucan enriched matrices have a protective effect and improve the growth of probiotic LAB (Pérez-Ramos et al., 2017a). Thus, the previous works support the potential of the *P. parvulus* 2.6 strain as a component of functional human food. In addition, we have previously shown that EPS-producing LAB isolated from meat fermented products have potential as fish probiotics and its EPS as antimicrobial and immunomodulator (Nácher-Vázquez et al., 2015, 2017). Therefore, *P. parvulus* 2.6 strain could also have interest for the fish feed sector.

Fish is a good food for humans, because its proteins are highly digestible, and it contains vitamins and minerals, and is the most important source of ω3 lipids, which are important for reducing cardiovascular risk (Marik and Varon, 2009). Wild fish is a limited resource on earth, but fish farms could assure the human supply in the future. To increase the profitability of aquaculture, one of the most important aspects is to reduce the cost of feeding. For this aim, in recent years, the use of feed with 100% vegetable proteins has obtained successful results. However, the use of these kinds of feeds present certain problems related to the fish immune system, affecting their intestinal morphology and producing diseases such as enteritis and diarrhea. These pathological problems can lead to death due to bacterial or viral outbreaks, which can cause considerable economic loss in aquaculture (Subasinghe, 2005; Torrecillas et al., 2007). To diminish this problem, β-glucans from plant, fungi, and yeast are widely utilized to avoid the outbreak of infectious diseases (Meena et al., 2013). Thus, the β-glucan produced by *P. parvulus* 2.6 as well as the bacterium itself could be of interest in aquaculture as an immunomodulant and as a probiotic, respectively. To prove these potential beneficial effects, its test in animal models is required. To this end, the zebrafish model has become a powerful tool for the study of vertebrate development, immunity, and diseases (Phelps and Neely, 2005; Goldsmith and Jobin, 2012; Novoa and Figueras, 2012). This is due to its high fecundity, small size, optical transparency at the beginning of the developmental stages, availability of genetic tools, and its immune system that possesses a high similarity to that of other vertebrates. Moreover, gnotobiotic zebrafish larvae are relatively easy to obtain and provide a good model to study bacterial colonization (Rendueles et al., 2012; Rieu et al., 2014; Russo et al., 2015) or host–pathogen interaction (Oyarbide et al., 2015) without interference of the gut microbiota or environmental microorganisms. In addition, the optical transparency of zebrafish larvae allows the easy detection of fluorescent-labeled bacteria for studies of host–microbe interactions (Rieu et al., 2014; Oyarbide et al., 2015; Russo et al., 2015). Thus, the development of fluorescence reporter systems to label putative probiotic bacteria is useful for tracking them inside the animal model used (van Zyl et al., 2015; Landete et al., 2016). Accordingly, we have developed the pRCR12 plasmid (Russo et al., 2015) derived from the pRCR promoter probe vector (Mohedano et al., 2015), which expresses constitutively the red fluorescent protein mCherry, it is useful for the fluorescent-labeling and detection of LAB, and it has been already validated in *Lactobacillus acidophilus*, *Lactobacillus casei*, *Lactobacilus fermentum*, *Lactobacillus plantarum*, *Lactobacillus sakei*, *Lactococcus lactis*, and *Streptococcus pneumoniae* (Mohedano et al., 2015; Russo et al., 2015; Nácher-Vázquez et al., 2017; Pérez-Ramos et al., 2017b). Moreover, the fluorescence labeling of *L. fermentum*, *L plantarum*, and *L. sakei* with pRCR12 has allowed visualization of these bacteria in the zebrafish gut (Russo et al., 2015; Nácher-Vázquez et al., 2017).

Thus, in this work, we have labeled *P. parvulus* 2.6 and its isogenic EPS-non-producing 2.6NR strains with the pRCR12 plasmid and zebrafish larvae models were used to perform *in vivo* a comparative study of the probiotic properties of these strains as well as of the immunomodulatory properties of the β-glucan produced by *P. parvulus* 2.6.

MATERIALS AND METHODS

Bacterial Strains, Growth Conditions, and Plasmid

The 2-substituted (1,3)-β-D-glucan-producing *P. parvulus* 2.6 and its isogenic non-producing strain *P. parvulus* 2.6NR (Fernández et al., 1995) were used in this work. *L. plantarum* 90[pRCR12] (Russo et al., 2015) was used as source of plasmid pRCR12 to electrotransform the *Pediococcus* strains. Plasmid pRCR12 carries a transcriptional fusion of the pneumococcal P_x promoter and the *mrfp* gene, whose codons are optimized for expression in LAB, and which encodes a monomeric version of the red fluorescent protein of *Discosoma* sp. (Garcia-Cayuela et al., 2012). LAB were routinely grown at 30°C in Man Ragosa Sharpe (MRS) medium (Pronadisa, Spain) supplemented with chloramphenicol (Cm) at 10 μg mL^{-1} for growth of strains carrying pRCR12 plasmid. *Escherichia coli* V517 is a multiple plasmid strain (Macrina et al., 1978) used in this work as standard to determine molecular weight of plasmids, and it was grown in LB broth (Pronadisa, Spain) at 37°C. The *Vibrio anguillarum* NB10[pOT11] serotype O1 strain used in this work (O'Toole et al., 2004) was kindly provided by R. O'Toole from Umeå University. This is a green fluorescent protein (GFP)-labeled bacterium due to the expression of the inducible *tac-gfpmut2* transcriptional fusion carried by the pOT11 plasmid. The bacterium was grown at 25°C in TSB (tryptic soy broth, Pronadisa) supplemented with Cm at 10 μg mL^{-1} and 0.5 mM isopropyl-β-D-thiogalactopyranoside to induce expression of the GFP protein.

Antibiotic Resistance Profile of *P. parvulus* 2.6 and 2.6NR Strains

The bacteria were screened for antibiotics resistance. The antibiotics recommended by the European Food Safety Authority (EFSA, 2012) to identify bacterial strains with potential acquired resistance to antibiotics were analyzed. The antibiotics tested were: ampicillin, chloramphenicol, clindamycin, erythromycin, gentamycin, kanamycin, streptomycin, tetracycline, and vancomycin. The minimal inhibitory concentration (MIC) was determined by the broth microdilution method reported by the ISO 10,932/IDF 233 standard (International Organization for Standardization, 2010). The strains were classified as susceptible or resistant according to the cut-off values proposed by EFSA (2012). A bacterial strain was defined as susceptible when its growth was inhibited at a specific antimicrobial concentration equal or lower than the established cut-off value and it was considered resistant when its growth was not inhibited at a concentration higher than the established cut-off value.

pRCR12 DNA Preparation and Transfer to *P. parvulus* Strains

The pRCR12 plasmid from *L. plantarum* 90 was isolated using the high pure plasmid isolation kit (Roche) as follows. The strain was grown to stationary phase [10^9 colony forming units (cfu) mL^{-1}] and 1 mL of the culture was sedimented by centrifugation at 10,000 × *g* for 10 min at 4°C. The cells were resuspended in solution I of the kit supplemented with lysozyme (30 mg mL^{-1}) and were incubated for 30 min at 37°C. Then, plasmid isolation was performed as described in the kit protocol, eluting the plasmidic DNA in 100 μL at approximately 100 ng μL^{-1}.

P. parvulus 2.6 and 2.6NR strains were electrotransformed with pRCR12 using the method of Berthier et al. (1996) with modifications, as follows. Bacterial cultures were grown in MRS supplemented with 40 mM D-threonine to an optical density at 600 nm ($OD_{600\,nm}$) of 0.8 (3 × 10^8 cfu mL^{-1}), sedimented by centrifugation at 5,500 × *g* for 10 min at 4°C, and the cells subjected to two cycles of resuspension in phosphate buffered saline (PBS) solution at pH 7.2 and sedimentation as above. In the case of the 2.6 strain, the bacteria were thoroughly vortexed during resuspension to remove its β-glucan from the bacterial surface to facilitate the subsequent incorporation of plasmidic DNA. Then, the cells were resuspended in 1 mL of lysozyme solution (final concentration of 2,000 U mL^{-1}) in PBS pH 7.2 and incubated for 20 min at 37°C. Afterward, bacterial cells were sedimented (3,300 × *g*, 5 min, 4°C) and subjected to cycles of resuspension in 1 mL (once in PBS pH 7.2, twice in 10 mM MgCl2) and once in electroporation buffer (0.5 M sucrose plus 10% glycerol) and sedimentation (3,300 × *g*, 5 min, 4°C), and the bacteria were resuspended in the electroporation buffer to a concentration of about 4 × 10^{10} cfu mL^{-1}. Then, 5 μL of pRCR12 plasmid (0.5 μg) was added to 50 μL of the bacterial suspension and were electroporated at 1.8 kV, 600 Ω, and 25 μF in a 0.2-cm cuvette using a Gene Pulser Xcell with ShockPod cuvette chamber (Bio-Rad, Hercules, CA, United States), obtaining a time constant of 12.5–13 ms. Transformants were selected in MRS-agar supplemented with Cm at 10 μg mL^{-1}. The obtained recombinant strains were designated 2.6[pRCR12] and 2.6NR[pRCR12].

Growth and Fluorescence Analysis of *P. parvulus* Strains 2.6[pRCR12] and 2.6NR[pRCR12]

Growth and red fluorescence emitted by the *P. parvulus* strains were monitored simultaneously with the Varioskan Flask System (Thermo Fisher Scientific, Waltham, MA, United States), which provides quantitative data of cell density by measuring the $OD_{600\,nm}$ and mCherry expression upon excitation at a wavelength of 587 nm and detection of emission at a wavelength of 612 nm. Overnight cultures were diluted in fresh medium to give an $OD_{600\,nm}$ = 0.1 and 300 μL of each culture was placed in triplicated in a sterile 96-Well Optical White w/Lid Cell Culture (Thermo Fisher Scientific, Rochester, NY, United States). The experiments were performed in triplicate incubating in the Varioskan at 30°C and measuring OD and fluorescence at 30 min intervals.

Fluorescence Microscopy and Transmission Electron Microscopy

Exponential cultures of *P. parvulus* strains and *V. anguillarum* NB10[pOT11] were sedimented and concentrated fivefold by resuspension in PBS pH 7.2. Then, without fixing, the suspension (10 μL) was used for phase contrast and fluorescent microscopy analysis with a Leica DM1000 model microscope (Leica Microsystems, Mannheim, Germany) with a light source EL6000 and the filter system TX2 ET and GFP ET for detection of mCherry and GFP fluorescences, respectively. The microscope was connected to a DFC3000G camera (Leica

Microsystems) with a CCD sensor. The image analysis was performed using Leica Application Suite X Software (Leica Microsystems).

Cultures of 2.6[pRCR12] and 2.6NR[pRCR12], prepared as described for binding to Caco-2 cells assay (see below), were used for electron microscopy analysis. A drop from each bacterial solution resuspended in 0.1 M $AcNH_4$, pH 7, was deposited on a carbon film copper grid, which had previously been hydrophilized by a glow discharge process for 1 min, and rinsed in water during 15 s. Then, the grid was stained during 10 s with a uranyl acetate water solution (0.2% w/v) in order to improve the image contrast, and finally, rinsed again in water. The sample was air-dried and examined in a JEOL JEM-1230 microscope (JEOL, Peabody, MA, United States), operating at an accelerating voltage of 100 kV.

Production, Purification, and Labeling of the 2-Substituted (1,3)-β-D-Glucan

The β-glucan of *P. parvulus* 2.6 was produced and purified by ethanol precipitation, dialysis, and chromatographic fractionation as previously described (Notararigo et al., 2013). 5-([4,6-Dichlorotriazin-2-yl] amino fluorescein hydrochloride (DTAF; Sigma-Aldrich) was used to fluorescently label the β-glucan; 1.6 mg of DTAF and 4 mg of β-glucan were dissolved in 2 mL of 0.1 M borate buffer, pH 9. The mix was incubated for 16 h at 25°C under an agitation of 600 rpm. The solution was dialyzed for 1 day against distilled water (changed twice), using a dialysis membrane having a cut-off of 12–14 kDa. After dialysis, the solution was frozen at −80°C, lyophilized, and kept at room temperature until its use for detection in zebrafish gut.

Quantification of the 2-Substituted (1,3)-β-D-Glucan

To determine the total concentration of the β-glucan produced by *P. parvulus* strains, bacteria were grown in MRS medium until late exponential phase ($OD_{600\,nm}$ = 3.0). Then, after vortexing to release the β-glucan attached to the bacteria, the bacteria were sedimented by centrifugation (16,000 × *g*, 10 min, 4°C), and the β-glucan present in the supernatants was quantified using a competition ELISA method (Werning et al., 2014). This method is based on the *S. pneumoniae* serotype 37 antibodies that can recognize specifically this bacterial β-glucan. The purified β-glucan of *P. parvulus* 2.6 was immobilized in each well of a 96-Well Nunc-Immuno MicroWell MaxiSorp plate (Thermo Fisher Scientific, Roskilde, Denmark) and used to compete with the β-glucan presented in the culture supernatants for binding to the primary antibody (antiserotype 37, Statens Serum Institut, Copenhagen, Denmark). The whole assay was carried out as previously described (Werning et al., 2014).

The ELISA method was also used to determine the concentration of β-glucan attached to the producing bacteria, with or without its removal, that were used for the adhesion assays to Caco-2 cells (see details above). Bacteria grown and treated as described above were resuspended in PBS pH 7.2 prior to direct determination of β-glucan concentration.

Plasmid Analysis and Detection of the *gtf* Gene by Southern Hybridization

With the aim of confirming the lack of plasmid rearrangements after transfer of pRCR12, the total plasmidic DNA of the *P. parvulus* parental and recombinant strains was obtained and location of the *gtf* genes was analyzed by Southern blot hybridization. Total plasmid DNA preparations of *P. parvulus* strains were obtained and purified by isopycnic CsCl density gradient to eliminate non-supercoiled forms of the plasmids as previously described (Pérez-Ramos et al., 2017b). Plasmid samples were fractionated in a 0.7% agarose gel and DNA molecules were revealed by staining with ethidium bromide at 0.5 mg mL^{-1}. The images of the gel were obtained with the GelDoc 200 equipment and the Quantity one 4.5.2 software (Bio-Rad, Laboratories Ltd., Alcobendas, Spain). For Southern blot hybridization, the DNA fragments were transferred to a nylon membrane as previously described (Pérez-Ramos et al., 2017b). An internal region of 598 bp of the *gtf* gene was amplified as previously described (Werning et al., 2006) by PCR with primers GTFSF (5′-TTGCCAGAACTAGAGAAAGTACGCA-3′) and GTFSR (5′-ACTTCCTATTTAGCTAAAAAGCAA-3′) using as substrate a total plasmidic DNA preparation of *P. parvulus* 2.6. The amplicon was labeled with digoxigenin-dUTP by using the DIG high prime DNA labeling and detection starter kit II (Roche, Mannheim, Germany) and the hybridization was performed as previously described (Pérez-Ramos et al., 2017b). The signals were detected with the LAS-3000 imaging system (Fujifilm, Stamford, CT, United States).

Adhesion of *P. parvulus* Strains to Caco-2 Cells

The Caco-2 human enterocyte cell line, obtained from the cell bank at CIB, was seeded in 96-well tissue culture plates (Falcon Microtest^{TM}, Becton Dickinson, Franklin Lakes, NJ, United States) at a final concentration of 1.25×10^5 cells mL^{-1} and grown as mono-layers of differentiated cells for 14 days as previously described (Nácher-Vázquez et al., 2017). Cell concentrations were determined as previously described (Garai-Ibabe et al., 2010).

To test the adhesion, overnight cultures of *P. parvulus* strains were diluted to give an $OD_{600\,nm}$ = 0.1 at 30°C and grown for 20 h, until late exponential phase. Then, samples of the *Pediococcus* strains were sedimented by centrifugation (9,300 × *g*, 10 min, 4°C), gently resuspended in PBS pH 7.2, and sedimented as before. In addition, with the aim of removing the EPS present on the surface of the *P. parvulus* 2.6[pRCR12] cells, a fraction of its bacterial culture designated 2.6p* was thoroughly vortexed prior to sedimentation as above and further subjected to two cycles of resuspension in PBS and sedimentation. Afterward, the bacterial samples were resuspended in Dulbecco's Modified Eagle Medium (DMEM, Invitrogen) at a concentration of 1.25×10^6 cfu mL^{-1} and added to the Caco-2 cells in a final volume of 0.1 mL per well. After incubation for 1 h at 37°C in an atmosphere containing 5%

CO_2, unattached bacteria were removed by three washing with 0.2 mL of PBS pH 7.2. Then, Caco-2 cells were detached from the well by incubating with 0.1 mL of 0.05% (w/v) trypsin-EDTA for 5 min at 37°C. The reaction was stopped by addition of 0.3 mL of PBS pH 7.2. To determine the number of cell-associated bacteria, appropriate dilutions were plated onto MRS-agar supplemented with Cm at 10 $\mu g\ mL^{-1}$. Three independent adhesion assays were performed in duplicate.

Animal Husbandry

Zebrafish embryos were obtained from wild-type adult zebrafish (*Danio rerio*, Hamilton, 1822), which were bred and maintained in the AZTI Zebrafish Facility (REGA number ES489010006105; Derio, Spain) as previously described (Russo et al., 2015) following standard conditions (Sullivan and Kim, 2008). During experimentation, the fish were maintained at an average density of 1.3 animals per mL in sterile petri dishes housed in an air incubator at 27°C on a 12 h light cycle. All experimental procedures were approved by the Regional Animal Welfare Body (Project ENVIPHAGE, NEIKER-OEBA-2015-004).

Production of germ-free zebrafish was performed as previously described (Oyarbide et al., 2015). Briefly, embryos were washed with a sterilized embryo wash buffer (EWB) solution [embryo water (EW) solution ($CaCl_2$ at 220.5 mg L^{-1}, $MgSO_4$ 7 H_2O at 92.5 mg L^{-1}, $NaHCO_3$ at 47.3 mg L^{-1}, and KCl at 4.1 mg L^{-1}) supplemented with methylene blue 0.01% (w/v)], antibiotic solution (kanamycin 15 $\mu g\ mL^{-1}$, ampicillin 300 $\mu g\ mL^{-1}$, and amphotericin B 1.25 $\mu g\ mL^{-1}$), and 0.02% (w/v) polyvinylpyrrolidone for 2 min. Then, washed with 0.003% (v/v) bleach solution for 1 h and finally washed with EWB solution. Afterward, the embryos were incubated overnight in antibiotic solution. The following day 50 embryos were collected and transferred to a Petri dish (5.5 cm diameter × 1.0 cm) to be immersed in 5 mL EWB solution and treated with two UV light pulses of 1.6 kV (Pulsed UV System XeMatica 1:2L-SA, SteriBeam Systems, GmbH) to inactivate bacteria present in the sample. The entire procedure was carried out inside a laminar flow cabinet to maintain sterile conditions; sterile solutions and materials were also used. Sterility was routinely tested after four days post fertilization (dpf), by culturing on general aerobic and yeast and molds culture media (Petrifilm aerobic, and Petrifilm yeast and molds count plates, 3 M).

Zebrafish Gut Colonization by *P. parvulus* 2.6[pRCR12] and 2.6NR[pRCR12] Strains

Cultures of *P. parvulus* strains were grown and treated in the same way as for the Caco-2 cells adhesion assays. Groups of 40 gnotobiotic zebrafish larvae at 4 dpf were placed into each Petri dish and were incubated with 30 mL of EW solution containing one of the bacterial strains at 5×10^7 cfu mL^{-1}. Larvae were incubated at 28°C with agitation (60 rpm) for 18 h. Then, the larvae were transferred to other sterile Petri dishes and the bacteria adhered to their surface were eliminated by washing them five times with PBS pH 7.2, and maintained in 30 mL of the same buffer. After, 6, 24, and 48 h post exposure (hpe), larvae were euthanized with tricaine at 300 mg mL^{-1}, and were individually visualized using a Leica MZFL III stereomicroscope (LeicaMicrosystems GmbH, Wetzlar, Germany) with a zoom magnification range of 8× to 100×. The microscope was equipped with a visible light and an ultraviolet light (Hg 100 W) source. mCherry fluorescence was detected by exposure of the larvae to ultraviolet light in the excitation range of 540–580 nm. Images were captured using a Leica DFC 360FX camera and processed with the LAS-AF (Leica Microsystems GmbH). In addition, three pools of five larvae of each treatment were washed twice in PBS containing 0.1% tween 20 to remove bacteria loosely attached to the skin, once with PBS pH 7.2, and finally were resuspended in 0.4 mL of the same buffer. Then, larvae were mechanically homogenized with a Pellet Pestle Cordless Motor (Kimble Chase Life Science and Research Products LLC), and the solutions obtained were plated onto MRS supplemented with Cm 10 $\mu g\ mL^{-1}$ plates. Bacterial cfu were counted after incubation at 30°C for 48 h. Three independent experiments were performed.

P. parvulus 2.6[pRCR12] and 2.6NR[pRCR12] Strains Activity Against *V. angillarum* NB10[pOT11] in the Zebrafish Model

Groups of 20 gnotobiotic zebrafish larvae at 4 dpf were pre-treated with either 2.6[pRCR12] or 2.6NR[pRCR12] strains as described above for 18 h. Then, the larvae were infected with bacterial solutions of *V. anguillarum* NB10[pOT11] previously grown during 16 h, sedimented by centrifugation (9,300 × *g*, 10 min, 4°C), and subjected to two cycles of resuspension in EW solution containing 0.5% NaCl and sedimentation as before. Finally, they were resuspended in EW to give a solution of 10^8 cfu mL^{-1}. Also, 20 gnotobiotic zebrafish larvae were infected with *V. anguillarum* without bacterial pre-treatment, and used as a positive infection control. In addition, 10 gnotobiotic zebrafish larvae were incubated in the absence of LAB and *V. anguillarum*, and used as a control of lack of mortality in the absence of infection. Larvae were individually placed into wells of 24-well plates with 1 mL of *V. anguillarum* solution. Bacterial solution was changed every 24 h and the infection was extended for 72 h. Mortality was examined at 24, 48, and 72 h post infection (hpi). Three independent experiments were performed in triplicate.

Antimicrobial Activity of *P. parvulus* Strains Against *V. anguillarum* Tested *in Vitro*

An agar spot test and a well diffusion assay were used, according to Schillinger and Lucke (1989). For the spot test, overnight cultures of *Pediococcus* strains were spotted (5 µL) onto the surface of MRS-agar plates, and incubated for 30 h at 30°C to allow colonies to develop. Approximately 5×10^7 CFU of *V. anguillarum* were inoculated into 10 mL of TSB-agar (containing 1% agar) and poured over the plates on which *Pediococcus* strains were grown. The plates were incubated for 24 h at 25°C and checked for inhibition zones. For the well diffusion assay, overnight cultures of *Pedioccocus* strains were sedimented by centrifugation (10,000 × *g*, 15 min at 4°C). Supernatants were recovered and the pH was measured. Then, an aliquot of the supernatants was adjusted to pH 6.5 with 3 M NaOH and sterilized by 0.22 μm pore-size Minisart® syringe filters (Sartorius stedim biotech, Gotinga, Germany). Approximately 5×10^7 CFU of *V. anguillarum* were inoculated into 10 mL of TSB-agar and poured over sterile plates. After solidification, wells of 3 mm of diameter were made in the agar plates; 60 μL of the supernatants with pH adjusted, or without, were placed into the wells; 0.1 M HCl and MRS medium were used as positive and negative controls, respectively. The plates were left at 4°C for 2 h to allow diffusion of the tested supernatants and then incubated for 24 h at 25°C. The absence and presence of inhibitory zones around the wells were recorded.

Immunomodulation of Gnotobiotic Zebrafish Larvae by the *P. parvulus* 2.6 β-Glucan

To show if the β-glucan enters inside of the zebrafish gut, five gnotobiotic larvae at 4 dpf were submerged in a solution of DTAF-labeled β-glucan at 150 μg mL^{-1} during 16 h. Then, the larvae were washed and after 6 h they were visualized using the Leica MZFL III stereomicroscope and the DTAF fluorescence was detected by exposure of the larvae to UV light in the excitation range of 450–490 nm. For the immunomodulation assay, groups of 20 gnotobiotic zebrafish larvae at 4 dpf were submerged in EW containing β-glucan at 150 μg mL^{-1} (treated) or in EW (control) solutions during 30 h. Then larvae were frozen in liquid nitrogen and total RNA was extracted using Trizol Reagent (Invitrogen Life Technology, Merelbeke, Belgium) according to the manufacturer's instructions. The quantity and quality of RNA samples were determined by capillary electrophoresis, using an Agilent 2100 Bioanalyzer (Agilent Technologies, las Rozas, Spain); 20 ng of each RNA sample was used to synthesize cDNA with the oligo $d(T)_{16}$ in a reverse transcription reaction with the TaqMan® Reverse Transcription kit (Applied Biosystems Life Technology, Belgium) and following the instructions of the manufacturer. Changes in mRNA expression of several genes, related to the innate immune system, were monitored by real-time qPCR, performed with SYBR Green PCR master mix (Roche Diagnostics, Rotkreuz, Switzerland) on a Roche LightCycler® 96 Instrument. The gene tested and the sequences of the primers used are listed in **Table 1**. The reaction conditions were: 95°C for 10 min followed by 40 cycles of 95°C for 10 s and 60°C for 30 s, and a dissociation step of 95°C for 1 min, 65°C for 1 min, and 95°C for 15 s. The mean C_T of each sample was normalized against the housekeeping genes (β-actin and elongation factor 1) and the corresponding control. The relative quantification of each gene was calculated by the $2^{-\Delta\Delta CT}$ method, using the REST 2009 software (Qiagen, Hilden, Germany).

Evaluation of *P. parvulus* 2.6 β-Glucan in a Model of Induced Inflammation of Zebrafish

The zebrafish transgenic line *Tg*(*mpx:GFP*)*i114* (Renshaw et al., 2006) whose neutrophils are GFP-labeled was used to make an induced inflammation model. β-glucan of the 2.6 strain purified as described in Section "Production, Purification, and Labeling of the 2-Substituted (1,3)-β-d-Glucan" was dissolved in EW solution at 150 μg mL^{-1}.The inflammation was induced on 14 larvae at 3 dpf by cutting off the apical region of their tails. Then, the larvae were divided into two groups and immediately individually submerged in EW containing β-glucan (treated) or in EW (control) solutions. Fluorescence images of each larva were taken at 0, 4, 8, and 24 h using the Leica MZFL III stereomicroscope and the GFP fluorescence was detected as indicated above for DTAF. The Images were processed and analyzed using the ImageJ 1.51w software (National Institute of Health, Maryland, United States). Three independent experiments were performed.

Statistical Analysis

In the Caco-2 cells adhesion assays as well as gut colonization and *Vibrio* infection analyses, the data are expressed as mean ± SD calculated from three independent experiments. The data were subjected to one-way analysis of variance (ANOVA) by using the SAS software. Tukey's test was employed to determine the significant differences between the variables at $p \leq 0.05$. In the induced inflammation analysis, the data are expressed in logarithms as a mean ± SD calculated from three independent experiments. The two-sample *t*-test was employed to determine the significant differences between the control group and the treated group at $p \leq 0.05$.

RESULTS

Construction and *in Vitro* Characterization of *P. parvulus* 2.6[pRCR12] and 2.6NR[pRCR12] Strains

As stated above, previous results indicated the probiotic potential of the 2-substituted (1,3)-β-D-glucan-producing *P. parvulus* 2.6 (reviewed in Pérez-Ramos et al., 2015). In addition, evaluation of this bacterium and its isogenic non-producing 2.6NR strain according with the EFSA specifications revealed that these bacteria were sensitive to all the antibiotics tested (Supplementary Table S1), and consequently there were not substrates for horizontal transfer of antibiotic resistance determinants. Therefore, we envisaged the *in vivo* evaluation of these pediococci in zebrafish models. Thus, with the aim of visualizing pediococci in the gut of the zebrafish larvae, the 2.6 and 2.6NR strains were fluorescently labeled with the mCherry protein by transfer of the pRCR12 plasmid. The resulting recombinant strains were designated 2.6[pRCR12] (2.6p) and 2.6NR[pRCR12] (2.6NRp) and total plasmid DNA preparations of these strains were compared with those of the parental strains by analysis in agarose gels (**Figure 1**) to confirm that the genetic manipulations have not affected the former plasmid content. *P. parvulus* 2.6 carries three natural plasmids: pPP1 (39.1 kbp), pPP2 (24.5 kbp), and pPP3 (12.7 kbp; Pérez-Ramos et al., 2017b), and the 2.6NR strain was generated from 2.6 by pPP2 plasmid curing (Fernández et al., 1995). Thus, according with these differences, the plasmidic DNA analysis revealed that 2.6p and 2.6NRp harbor the expected plasmids and

TABLE 1 | Oligonucleotides used in qPCR reactions.

Primers	Sequence (5′–3′)	Gene	NCBI ID
ACT-F	TGCTGTTTTCCCCTCCATTG	Beta actin	NM_131031.1
ACT-R	TTCTGTCCCATGCCAACCA		
EF1-F	GCCAACTTCAACGCTCAGG	Elongation factor 1	NM_131263.1
EF1-R	AGAGATCTGACCAGGGTGGTTC		
IL1B-F	CATTTGCAGGCCGTCACA	Interleukin 1-β	NM_212844.2
IL1B-R	GGACATGCTGAAGCGCACTT		
IL8-F	CCCTCTGCTCCATGGGTTAA	Interleukin 8 (Chemokine 8)	XM_001342570.3
IL8-R	CAGGTGATCCGGGCATTC		
MyD88-F	CACAGGAGAGAGAAGGAGTCACG	Myeloid differentiation primary response gen 88	NM_212814.2
MyD88-R	ACTCTGACAGTAGCAGATGAAAGCAT		
NFKB-F	AGAGAGCGCTTGCGTCCTT	Nuclear factor kappa B	NM_001003414.1
NFKB-R	TTGCCTTTGGTTTTTCGGTAA		
TLR2-F	GGAAGGTGGCACTAAGAGCCT	Toll-like receptor 2	NM_212812.1
TLR2-R	TGATCGGTCGTGGAGGAGTT		
TLR22-F	CCAGCTCTCGCCGTACCA	Toll-like receptor 22	NM_001128675.2
TLR22-R	TTGGGCCAGCGGATGT		
TLR4-F	GGGAAGTCAATCGCCTCCA	Toll-like receptor 4	NM_001121051.1
TLR4-R	ACGGCTGCCCATTATTCCT		
IL10-F	ATATTTCAGGAACTCAAGCGGG	Interleukin 10	NM_001020785
IL10-R	ACTTCAAAGGGATTTTGGCAAG		
TNFa-F	ACCAGGCCTTTTCTTCAGGT	Tumor necrosis factor alpha	NM_212859.2
TNFa-R	GCATGGCTCATAAGCACTTGTT		

that these bacteria also carry the 4.4 kbp pRCR12 plasmid. In addition, the plasmidic profiles showed that the establishment of pRCR12 in these hosts did not modify significantly the ratios of the natural plasmids. Consequently, it seems that there is no incompatibility between the replication machinery of pRCR12 plasmid and those of the other plasmids. The pPP2 plasmid harbors the *gtf* gene, which encodes the GTF glycosyltransferase responsible for the β-glucan synthesis (Werning et al., 2006). Therefore, we searched for the presence of the *gtf* gene in the plasmidic DNA samples. A Southern blot hybridization of the total plasmidic DNA preparations of the four *P. parvulus* strains was performed using an internal region of the *gtf* gene as a probe. As expected, hybridization signals were observed at the position of the 24.5 kbp pPP2 plasmid only in the 2.6 and 2.6p DNA samples (**Figure 1**).

With regard to the expression of the mCherry fluorescent protein, *P. parvulus* 2.6p and 2.6NRp formed bright pink colonies on MRS-agar medium (**Figure 2A**), and this coloration was visible even after incubation of the plates for 16 days (Supplementary Figure S1). This long-term detection indicated the stability of mCherry labeling in these hosts. Moreover, the colonies of the 2.6p strain retained the ropy phenotype of its parental 2.6 strain (**Figure 2A**) indicating that both produce the 2-substituted (1,3)-β-D-glucan. To validate this hypothesis, supernatants of cultures of both pediococci were used to determine the concentration of EPS released to the medium by these strains, using an immunological method for specific detection of the pediococcal β-glucan. The results revealed a similar production of the EPS, e.g., at an $OD_{600\ nm} = 1.0$, 114.97 ± 2.58 and 130.95 ± 6.53 mg L^{-1} for the 2.6 and 2.6p strains, respectively. In addition, expression of the mCherry protein was visualized at the cellular level by fluorescence microscopy. This analysis revealed an intense red color of the 2.6p and 2.6NRp bacteria, and superimposition of phase contrast and fluorescent images showed that all bacteria of both populations were expressing active mCherry protein (**Figure 2B**).

The use of the recombinant strains 2.6p and 2.6NRp instead of 2.6 and 2.6NR required that the genetic manipulation did not drastically affect the bacterial growth. Thus, the growth of the four *P. parvulus* strains in liquid medium was analyzed and the growth rate at the exponential phase was calculated (**Figure 2C**). The β-glucan-non-producing 2.6NR strain displayed a higher growth rate ($\mu = 0.231 \pm 0.024$) than that of the 2.6 strain ($\mu = 0.182 \pm 0.005$). The same behavior was observed, when the growth rate of the recombinant strains was compared $\mu = 0.184 \pm 0.013$ for 2.6NRp strain and $\mu = 0.174 \pm 0.011$ for 2.6p strain, but less differences between them were observed, a good characteristic for further comparative studies of bacteria–zebrafish interactions. In addition, the 2.6 and 2.6NR strains grew slightly faster than the corresponding recombinant strains. This behavior was expected, since only 2.6p and 2.6NRp were grown in the presence of Cm, because the pRCR12 plasmid confers resistance to this antibiotic. The high level of expression of the mCherry in the recombinant pediococci made it possible to analyze in real time, and simultaneously the growth (**Figure 2C**) and the fluorescence (**Figure 2D**) of the cultures. The levels of the red fluorescence emitted by the recombinant strains were increasing during the bacterial growth and remained stable during the stationary phase. These were similar in both strains and achieved a value of

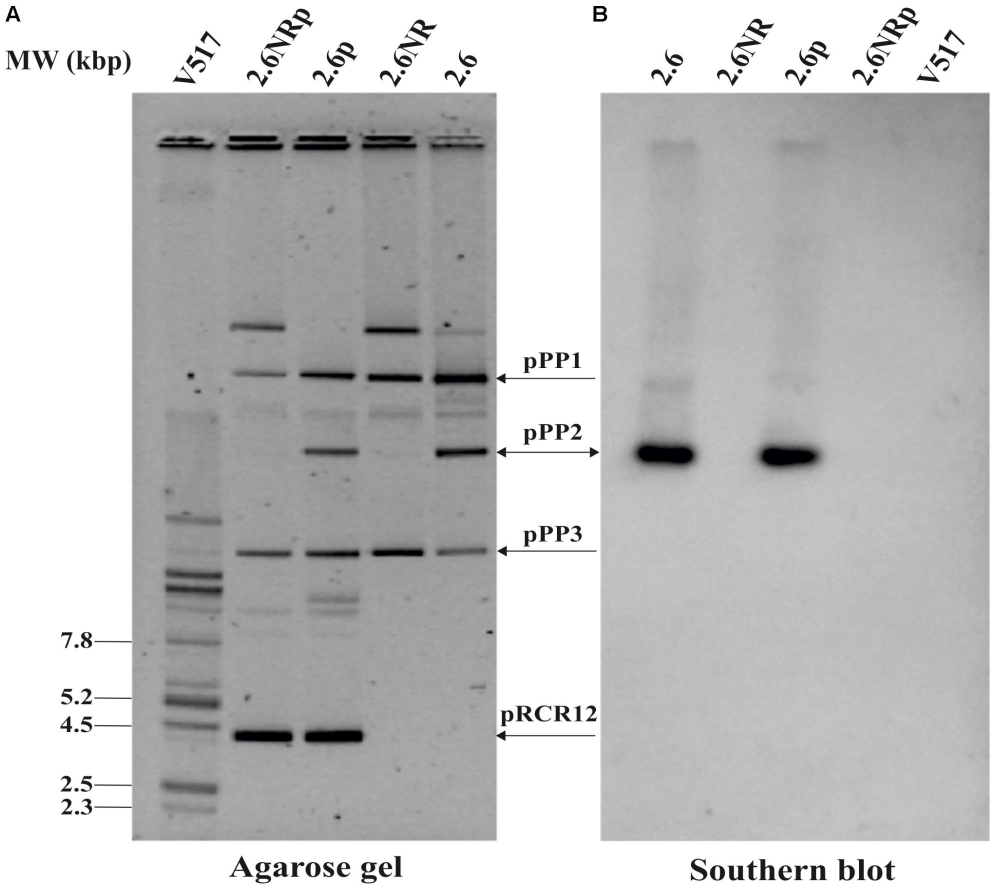

FIGURE 1 | Detection of *P. parvulus* plasmids and *gtf* gene. Plasmids' preparations of *P. parvulus* 2.6, 2.6NR, 2.6p, and 2.6NRp strains and of *E. coli* V517 were fractionated by electrophoresis in a 0.7% agarose gel, transferred to a nylon membrane, and the *gtf* gene detected by Southern blot hybridization. **(A)** The gel. **(B)** The hybridized membrane. The headed and double headed arrows indicate the positions of the covalently closed monomeric forms of the plasmids and the position of the hybridized bands in the corresponding gel, respectively.

111.27 ± 7.03 for 2.6NRp and of 93.18 ± 2.85 for 2.6p at $OD_{600\ nm} = 1.0$.

The capacity to adhere to the intestinal epithelium is one of the properties sought in probiotic bacteria. Therefore, we have previously investigated adhesion of *P. parvulus* 2.6 and 2.6NR strains to colon Caco-2 human cells (Fernández de Palencia et al., 2009), and detected a positive influence of the β-glucan in bacterial–human cells interaction. In this work, we had analyzed binding to these enterocytes of 2.6p with (called 2.6p*) and without mechanical removal of its β-glucan and 2.6NRp alone or supplemented with *P. parvulus* 2.6 purified β-glucan (called 2.6NRp+EPS; **Figure 3**). In these types of experiments, prior to expose to the eukaryotic cell, the growth medium of the bacteria has to be removed by centrifugation. Thus, the β-glucan that remains attached to the bacteria after sedimentation and resuspension as well as after mechanical removal was quantified with the specific ELISA method. The results revealed that during the adhesion assay, the EPS bound to the 2.6p and 2.6p* samples, was at a concentration of 248 ± 11 and of 3.7 ± 0.1 ng mL^{-1}, respectively. Moreover, as expected, the immunological assay revealed no detection of the β-glucan bound to 2.6NRp strain. Furthermore, analysis of the bacteria by transmission electronic microscopy confirmed the presence of the EPS bound to the 2.6p cells, the almost complete removal in the 2.6p*, and its absence in 2.6NR (insets in **Figure 3**). The results of the binding assays showed that the 2.6p strain had a level of adhesion to Caco-2 cells significantly higher than that achieved by the EPS-non-producing strain 2.6NRp (4.7 *versus* 0.4%). These values of binding were similar to those of the parental 2.6 and 2.6NR strains (6.1 *versus* 0.2% and 4.7 *versus* 0.7%, respectively, in Fernández de Palencia et al. (2009) and in Supplementary Figure S2). Moreover, when the β-glucan of 2.6p was substantially reduced, the adherence of this bacterium was similar to that of the 2.6NRp strain (0.6 *versus* 0.4%). Conversely, when the β-glucan was added to the 2.6NRp strain, the adherence of this bacterium increased considerably (2.2 *versus* 0.4%), confirming that the β-glucan is directly involved in the adherence of *P. parvulus* to enterocytes. Therefore, the overall results demonstrate the suitability of the 2.6p and 2.6NRp strains, rather than the parental strains to perform validated *in vivo* studies of zebrafish larvae–pediococci interactions.

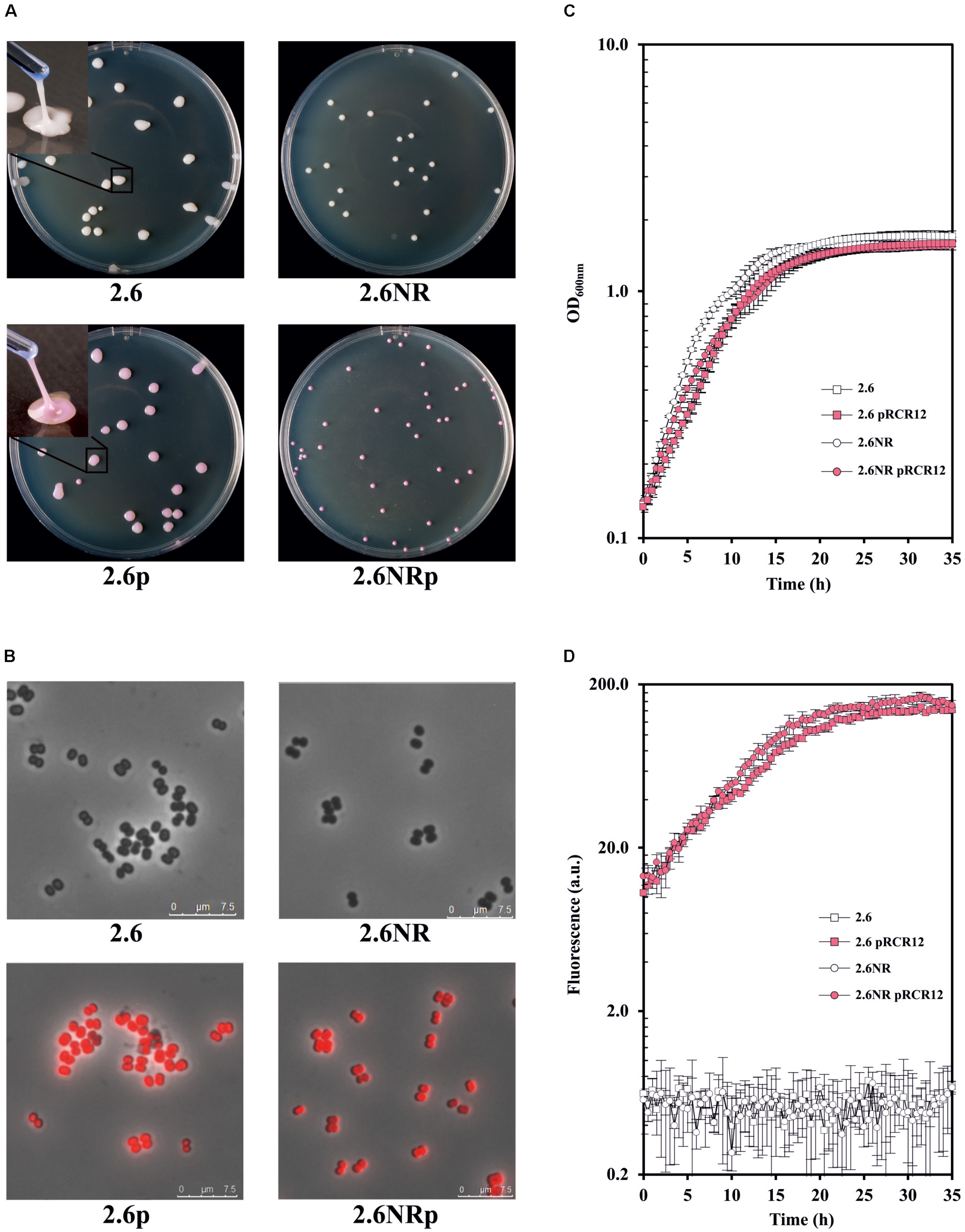

FIGURE 2 | Comparative analysis of the *P. parvulus* 2.6, 2.6NR, 2.6p, and 2.6NRp strains. **(A)** Colony phenotypes of the recombinant and their parental strains as well as colony lifting of the β-glucan-producing strains. Visualization of bacteria by optical microscopy with an objective of 100×. **(B)** Overlays of phase contrast and fluorescence images of the four *P. parvulus* strains. Analysis of bacterial growth **(C)** and the red fluorescence emission **(D)** of the four *P. parvulus* strains measured with the Varioskan Flask System.

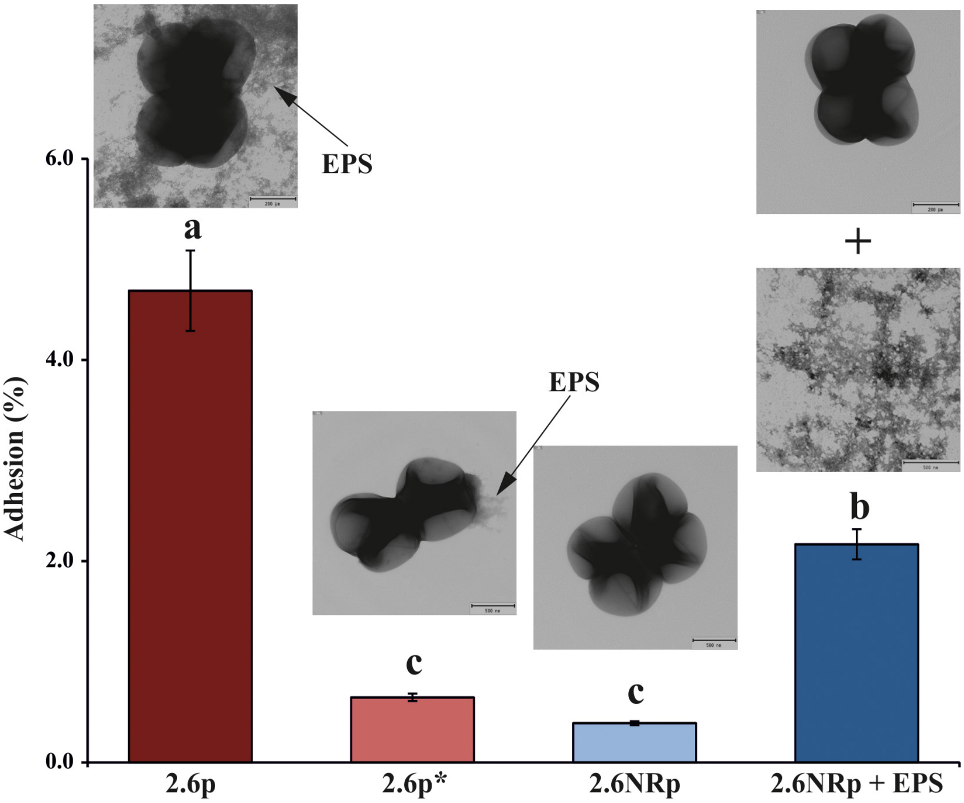

FIGURE 3 | Bacterial adhesion to a monolayer of Caco-2 cells. Enterocytes were exposed independently to four bacterial solution containing: (i) 2.6p strain, (ii) 2.6p* treated to remove most of the β-glucan, (iii) 2.6NRp, and (iv) 2.6NRp plus the amount of purified β-glucan detected in 2.6p strain solution. The insets show electron micrographics of the bacteria. The arrows mark the β-glucan (EPS). Statistical significances are represented by different letters that mean $P \leq 0.01$.

In Vivo Evaluation of Probiotic Properties of *P. parvulus* 2.6p and 2.6NRp Strains in Zebrafish Model

Animal models are necessary to validate the probiotic properties of bacteria. Currently, the zebrafish models are used for this purpose (Rendueles et al., 2012; Russo et al., 2015; Nácher-Vázquez et al., 2017). Thus, the colonization capacity of the 2.6p and 2.6NRp strains was tested by their exposure to gnotobiotic zebrafish larvae and quantified by plating the bacteria present in the intestinal tract of the larvae according to the protocol depicted in **Figure 4A**. For the two strains and in all conditions tested, the prevalence in the zebrafish gut decreased with the time of incubation (**Figure 4B**). In addition, at all the times tested (6, 24, and 48 hpe to the bacteria), the results corroborated *in vivo* the positive influence of the β-glucan in adherence capability of *P. parvulus* 2.6p. The colonization by this bacterium was significantly higher than that of the EPS-non-producing strain 2.6NRp (11.4, 7.1, and 2.8% *versus* 1.5, 0.6, and 0.5% at 6, 24, and 72 hpe, respectively). In addition, removal of the β-glucan decreased the colonization levels of the 2.6p strain to values (3.1, 2.2, and 1.2% at 6, 24, and 72 hpe, respectively) no significantly different to that of the 2.6NRp. Furthermore, these results correlated with those obtained in the bacteria–Caco-2 cells' interaction studies (**Figure 3**). Moreover, the mCherry-labeling of the *P. parvulus* strains allowed monitoring the zebrafish gut colonization by fluorescence microscopy. In **Figure 5**, are depicted representative images of 2.6p and 2.6NRp strains on larvae intestinal epithelium. In both cases, the levels of fluorescence decreased with the incubation time and they were higher in the case of 2.6p.

Also, the influence of *P. parvulus* colonization against infection by *V. anguillarum* was investigated. To this end, gnotobiotic zebrafish larvae previously colonized by either *P. parvulus* 2.6p, 2.6p*, or 2.6NRp strains were infected with *V. anguillarum* NB10[pOT11] according to the protocol depicted in **Figure 6A**. The results showed 100% of larvae survival in the absence of treatment with bacteria (data not shown). In addition, they revealed that both 2.6p and 2.6NRp strains were able to decrease significantly the larvae mortality due to *Vibrio* infection, being more pronounced the effect of 2.6p without EPS removal (**Figure 6B**). The protective effect was extended during the entire infection assay (**Figure 6B**). Thus, at 72 hpi, a survival of only 17.2% was detected in the control group infected with *V. anguillarum* and not pre-treated with LAB, whereas in the 2.6p-colonized group, a 60.6% of alive larvae was observed. Furthermore, the groups colonized by 2.6p* and 2.6NRp achieved an intermediate degree of survival, 45.5 and 36.1%, respectively. Therefore, colonization by the LAB seems to play a role in the

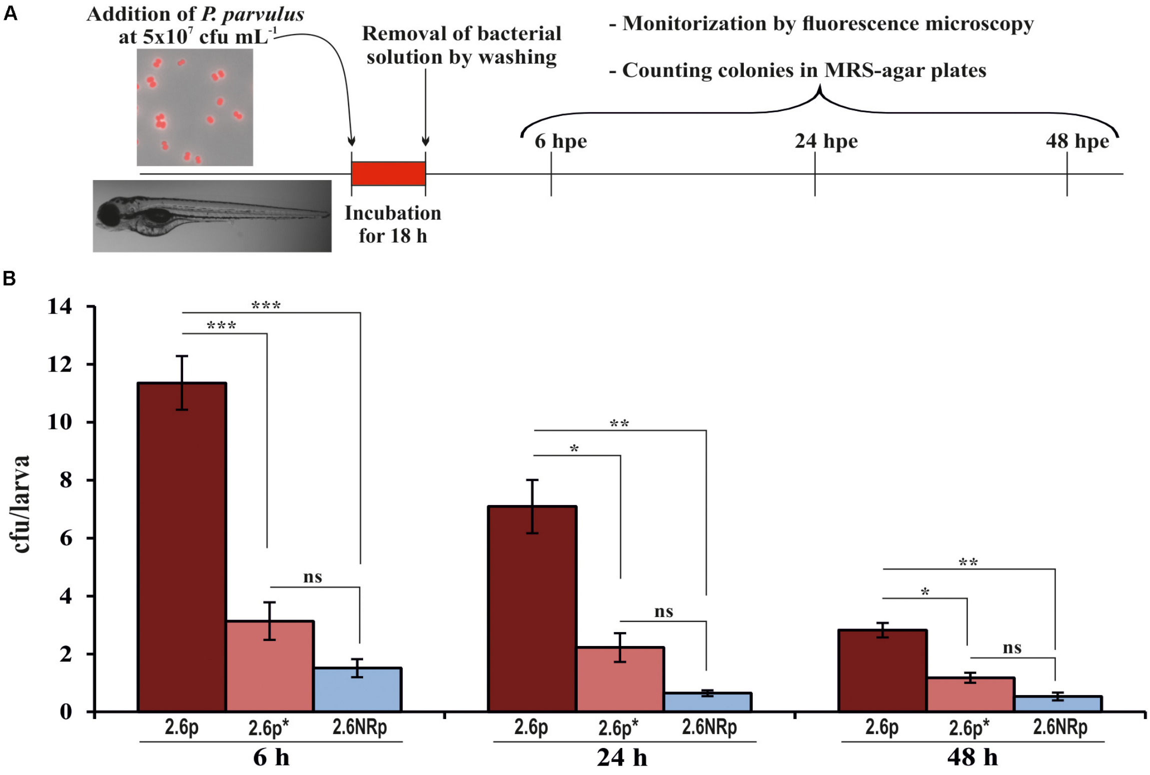

FIGURE 4 | Zebrafish gut colonization by *P. parvulus* 2.6p and 2.6NRp strains. **(A)** The experimental procedure is depicted. Four-days-old larvae were colonized during 18 h by 2.6p with (2.6p*) or without β-glucan removal or 2.6NRp strains. **(B)** The pediococcal colonies presented inside the larvae were analyzed at 6, 24, and 48 h after the bacteria were removed from the incubation buffer (hours post exposure, hpe). Differences between conditions were evaluated at each point time. Statistical significances are represented by $^*P \leq 0.05$, $^{**}P \leq 0.01$, $^{***}P \leq 0.001$, and ns ($P > 0.05$).

inhibition of pathogenic effect of *V. anguillarum* NB10[pOT11]. Thus, the overall results support the potential use of *P. parvulus* 2.6 as probiotic to prevent fish infections.

Immunomodulatory Properties of the 2-Substituted (1,3)-β-D-Glucan in Zebrafish Models

The zebrafish is currently used for immunomodulation studies, since its innate immunological response is similar to that of other vertebrates (Novoa and Figueras, 2012; de Oliveira et al., 2013). Thus, the purified β-glucan of *P. parvulus* 2.6 could be evaluated as an immunomodulator in zebrafish models.

Prior to this evaluation, the capability of gnotobiotic zebrafish larvae to ingest the β-glucan was tested. The purified EPS was green-fluorescently-labeled with DTAF and, 6 h after exposure, fluorescence was detected in the zebrafish gut by fluorescence microscopy (**Figure 7A**). Afterward, the unlabeled *P. parvulus* 2.6 β-glucan was evaluated in two zebrafish models. First, it was studied its effect on the immune system of non-stimulated zebrafish larvae. Gnotobiotic larvae at 4 dpf were submerged in β-glucan solutions at 150 μg mL^{-1} for 30 h, after that variation on gene expression of nine immune related genes was evaluated (**Figure 7C**) according with the protocol depicted in **Figure 7B**. The exposure of larvae to the β-glucan caused the downregulation of three genes involved in inflammatory response. Expression of two pro-inflammatory cytokine genes, IL8 and TNFα, was repressed 3.4-fold and 2.2-fold, respectively, and the adapter protein gene MyD88 was repressed 2.4-fold. In addition, the expression of anti-inflammatory cytokine gene IL10 showed a non-significant tendency to increase. The rest of the genes evaluated did not show any change in their expression levels.

In addition, the β-glucan was evaluated in an induced inflammation model using the zebrafish transgenic line *Tg*(*mpx:GFP*)*i114*. In this line, the neutrophils are GFP-labeled, allowing their detection during the inflammation response. The inflammation in the larvae was induced by cutting the apical region of the tail. Then, the recruitment and proliferation of the neutrophils in the larvae, during the first 24 h of the inflammation process, was visualized (**Figure 8A**) and quantified (**Figure 8B**) by fluorescence microscopy and images analysis, respectively. Quantification of the GFP levels at the tail region revealed that in the control larvae group, not exposed to the β-glucan, the recruitment of the neutrophils in the inflammation area drastically increased (9.4 logs) during the first 4 h and remained at this level (9.6 log) even after 24 h. However, the larvae treated with the β-glucan showed a lower recruitment of neutrophils with an increase of 8.0 logs during the first 4 h, that was further reduced to 7.3 logs after 24 h. Attending to the whole fluorescence signal in larvae, what is representative

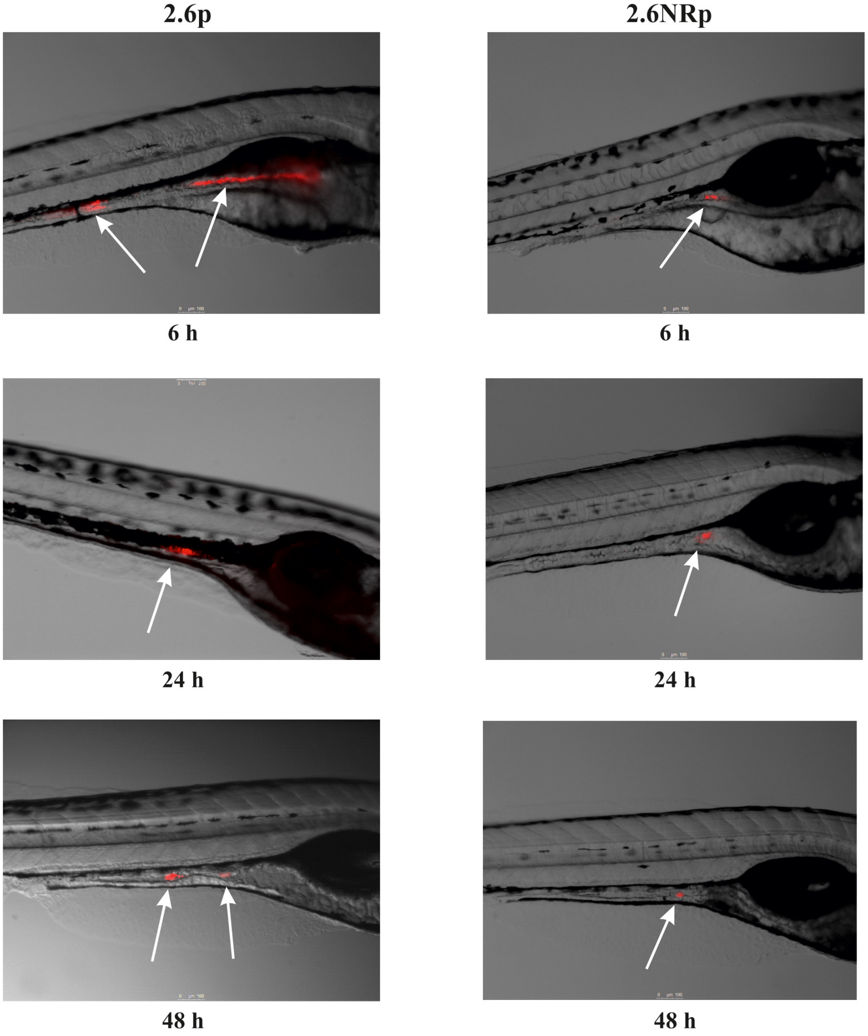

FIGURE 5 | Fluorescence microscopy images of zebrafish gut colonization by *P. parvulus* 2.6p and 2.6NRp strains. Representative images taken at 6, 24, and 48 hpe are depicted. White arrows mark the red fluorescence signal emitted by the bacterial cells inside the zebrafish gut.

of neutrophil proliferation, there were also differences between the control and the treated larvae groups after 24 h, when the fluorescence increases were 7.2 and 5.8 logs, respectively. These results indicated that in zebrafish larvae subjected to an inflammatory process, the presence of the β-glucan provoked an anti-inflammatory response manifested by a decreasing of the recruitment and proliferation of the neutrophils.

DISCUSSION

Disease control and prevention strategies in aquaculture have been based primarily on the use of antibiotics. However, this indiscriminate use can lead to major drawbacks such as the emergence of resistant strains, contamination of the aquatic environment, toxicity to the organism, and the accumulation of residues in organs and tissues, as well as other potential negative effects on human health. For this reason, it is necessary to drastically reduce the use of antibiotics in aquaculture (Cabello, 2006; Defoirdt et al., 2011). On the other hand, the normal microbiota of the digestive tract of fish plays a fundamental role in the exclusion of pathogens and in the maintenance of health. In this way, the use of probiotic bacteria (Newaj-Fyzul et al., 2014; Hai, 2015) can improve the feed conversion efficiency and live weight gain. In addition, probiotics can confer protection against pathogens by competitive exclusion for adhesion sites, production of organic acids (formic acid, acetic acid, and lactic acid), hydrogen peroxide, bacteriocins, or lysozyme and also modulate physiological and immunological responses in fish (Nayak, 2010). Thus, the use of probiotic bacteria is another strategy for improving production in fish farms.

Consequently, in this work, we have characterized the probiotic properties of the 2-substituted (1,3)-β-D-glucan-producing *P. parvulus* 2.6 in comparison with the non-producing *P. parvulus* 2.6NR strain to evaluate its potential as a probiotic in aquaculture, upon mCherry-labeling by transfer

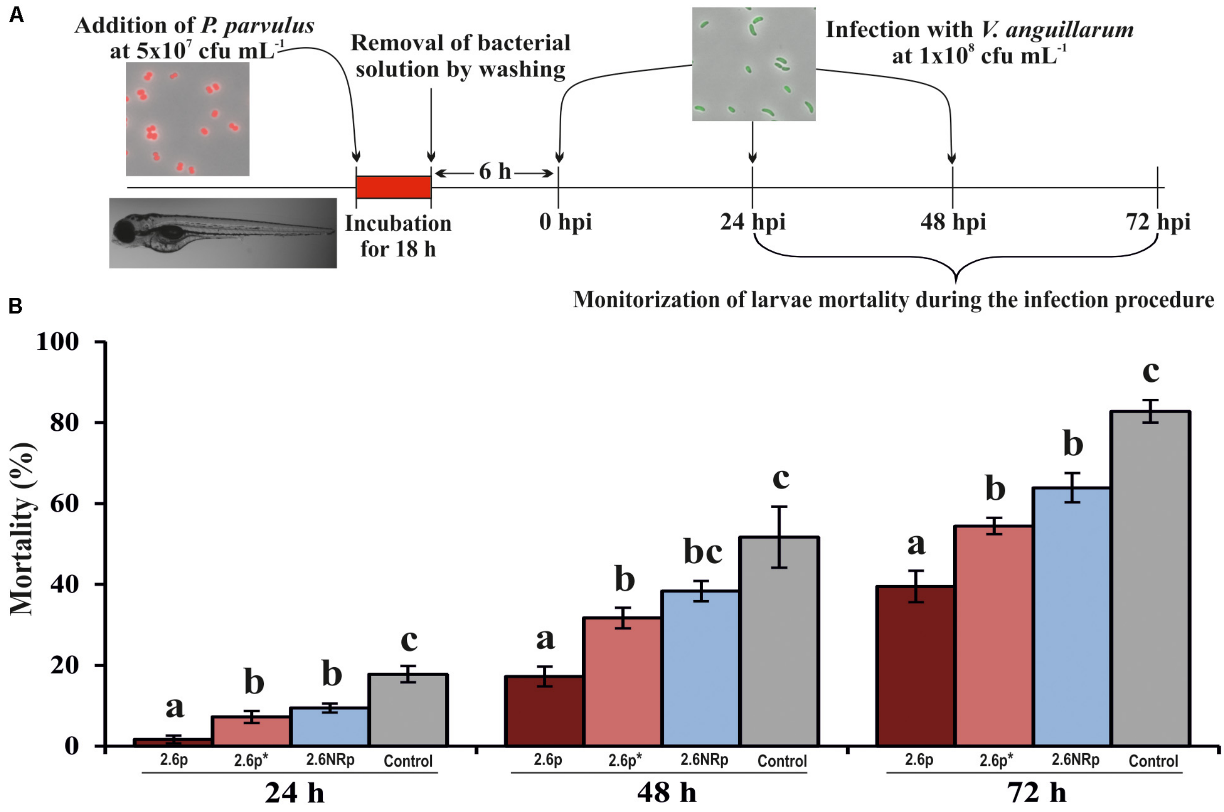

FIGURE 6 | Protective effect of *P. parvulus* 2.6p and 2.6NRp strains against *V. anguillarum* NB10[pOT11] zebrafish infection. **(A)** The experimental procedure. Before infection with *V. angillarum*, 4-days-old larvae were colonized during 18 h either by 2.6p with (2.6p*) or without β-glucan removal or by 2.6NRp strains. Also, as a control, a group of larvae was incubated in the absence of bacteria during 18 h prior infection. **(B)** Cumulative mortality due to *Vibrio* infection was analyzed at 24, 48, and 72 h after addition of the pathogen (hours post infection, hpi). Differences between conditions were evaluated at each point time. Statistical significances are represented by different letters that mean $P \leq 0.05$.

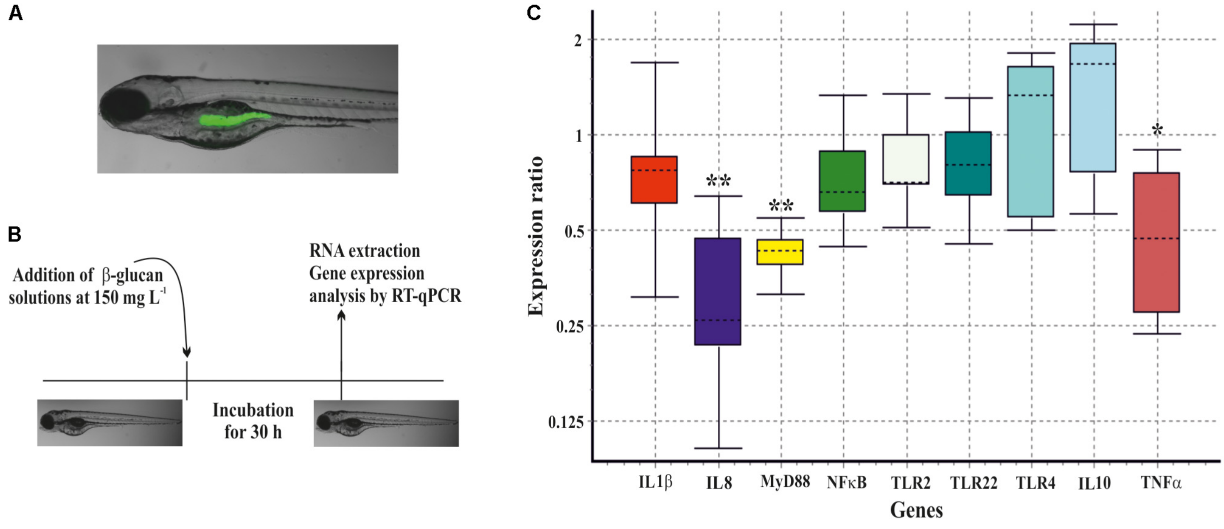

FIGURE 7 | Immunomodulation of gnotobiotic zebrafish larvae by the *P. parvulus* 2.6 β-glucan. **(A)** DTAF-labeled β-glucan was detected inside the zebrafish gut. **(B)** The experimental procedure for the immunomodulation assay. **(C)** Relative expression values of the genes involved in inflammation. The genes actin-β and elongation factor-1 were used as housekeeping to normalize the values. Statistical significances are represented by $^*P \leq 0.05$ and $^{**}P \leq 0.01$.

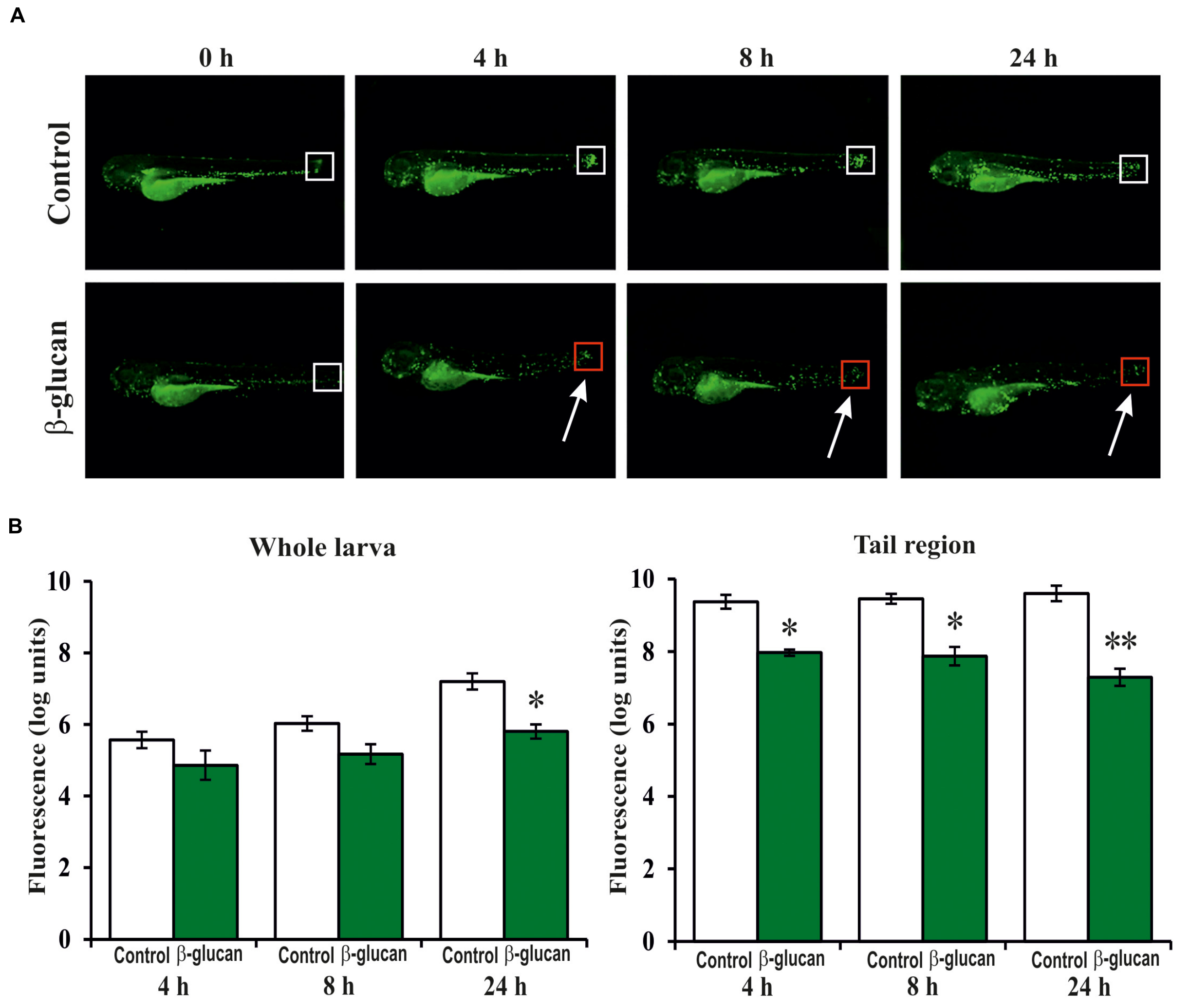

FIGURE 8 | Anti-inflammatory effect of the *P. parvulus* 2.6 β-glucan in an induced inflammation model of the *Tg*(*mpx:GFP*) zebrafish line. The inflammation was induced by cutting off the apical region of the tail. **(A)** Images of representative larvae treated and untreated with the β-glucan are depicted. Squares mark the areas of inflammation, and the colors indicate regions (red) with less migration of neutrophils than others (white). **(B)** The green fluorescence emitted by the neutrophils was quantified in the whole larvae and in their tails regions. The fluorescence values detected during the inflammation procedure were expressed as increased percentages of the values detected at 0 h, and represented as logarithms. Statistical significances between groups are represented by $^{*}P \leq 0.05$ and $^{**}P \leq 0.01$.

of pRCR12 plasmid. There are a very few works reporting electrotransformation of strains belonging to the *Pediococcus* genus, e.g., transfer of derivatives of pRS4 plasmid of *Pediococcus pentosaceus* (Alegre et al., 2005) by electroporation to *Pediococcus acidilactici* (Rodríguez et al., 2007). In addition, as far as we know this is the first report of plasmid transfer to and fluorescence-labeling of *P. parvulus* strains, which did not significantly affect bacterial growth and β-glucan production.

Prior having a beneficial effect in the digestive tract, probiotic bacteria must resist among others to the stress due to the digestive enzymes. Previously, the survival of *P. parvulus* 2.6 and 2.6NR strains under an *in vitro* gastrointestinal model system was demonstrated (Fernández de Palencia et al., 2009), but no influence of the β-glucan in resistance to the gut stresses was observed. However, when the *P. parvulus* 2.6 β-glucan was expressed in *Lactobacillus paracasei*, the presence of this EPS increased the bacterial survival to gastrointestinal stresses (Stack et al., 2010). In addition, the same effect of the polymer was observed, when the survival of *P. parvulus* 2.6 and 2.6NR strains through the digestive tract was evaluated using an *in vivo* mice model (Lindström et al., 2012). Accordingly, the results presented here revealed that *P. parvulus* 2.6 and 2.6NR remained viable inside the zebrafish gut, and this viability was improved in the presence of the β-glucan.

Many of the health effects exerted by the probiotic bacteria are related to their capability to adhere to the intestinal cells. Therefore, this is one of the main criteria for the selection of probiotic strains. In general, the scientific reports support that synthesis of EPS by LAB decreases the adhesion ability of the producing bacteria to enterocytes. This is the case of heteropolysaccharides of *Lactobacillus rhamnosus* (Lebeer et al., 2009; Polak-Berecka et al., 2014) and *Lactobacillus johnsonii* (Horn et al., 2013; Dertli et al., 2015) as well as dextrans (α-glucan homopolysaccharides) produced by *L. sakei* and *Leuconostoc mesenteroides* strains (Nácher-Vázquez et al., 2017; Zarour et al., 2017). However, Živković et al. (2016) detected a positive influence of an heteropolysaccharide of *L. paracasei* in bacterial adhesion to epithelial intestinal cells. Furthermore, we have demonstrated *in vitro* that the presence of the 2-substituted (1,3)-β-D-glucan promotes adhesion of *P. parvulus* strains to enterocytes (Fernández de Palencia et al., 2009; Garai-Ibabe et al., 2010; and this work). Moreover, the results presented here support that the presence of this EPS enhances *in vivo* the capabilities of *P. parvulus* for colonization of the zebrafish intestinal tract, by contrast with the dextran from *L. sakei*, which diminish the stay of the bacteria in the fish gut (Nácher-Vázquez et al., 2017). Also, the results obtained in this work suggest a transient colonization of the zebrafish gut by *P. parvulus* 2.6 strain. This behavior is expected for a probiotic bacteria, that by definition should be administrated daily (Guarner et al., 2017), and it has been previously detected with this zebrafish model for *L. plantarum*, *L. fermentum*, and *L. sakei* probiotic strains (Russo et al., 2015; Nácher-Vázquez et al., 2017).

Other property described for probiotic bacteria is to compete with pathogens involved in infection diseases, which are frequent in fish farms. Thus, in the last few years, there have been several reports concerning to the beneficial effect of probiotic candidates against various bacterial infections (Ran et al., 2012; Saini et al., 2014; Mohideen and Haniffa, 2015). Among the fish pathogens, *V. anguillarum* causes a deadly hemorrhagic septicemia disease named vibriosis, which provokes high morbidity and mortality rates and it is responsible for severe economic losses (Frans et al., 2011). In this context, we have previously showed that *L. sakei* MN1 competes with *V. anguillarum* in the zebrafish digestive tract (Nácher-Vázquez et al., 2017) and the results presented here revealed that pre-treatment with *P. parvulus* 2.6 has even a higher protective effect that *L. sakei* against vibriosis. Competition of probiotic with pathogenic bacteria could be due to either displacement, blocking their interaction with the epithelium, or stimulation of the immune system. In the case of *P. parvulus*, our results demonstrated that its β-glucan significantly contributes to its ability to compete with *V. anguillarum*, presumably by blocking the interaction of the pathogen with the enterocytes, and not by its displacement, since the zebrafish larvae were pre-treated with the LAB. However, the results presented here also suggest that the immunomodulation provoked by the *P. parvulus* 2.6 β-glucan (see details below) could contribute to the protective effect of the bacteria against *V. anguillarum* infection. Moreover, a killing of the pathogen by *P. parvulus* due to the LAB synthesis of antimicrobial compounds could take place, and in fact, there was *in vitro* detection of growth inhibition of a *V. anguillarum* lawn, when the pathogen was exposed to *P. parvulus* 2.6, 2.6p, 2.6NR, or 2.6NRp strains (Supplementary Figure S3A). This effect is presumably due to lactic acid secreted by the LAB, since exposure of the pathogen to the acidic (pH 4.0) bacterial culture supernatants yielded a small inhibition halo, which was not detected after the supernatants neutralization to pH 6.5 (Supplementary Figures S3B,C). However, this does not seem to be the major cause of the anti-infective activity of *P. parvulus* 2.6, because the same inhibition pattern was observed with the four strain tested. Nevertheless, independently of the mechanisms involved in the performance of *P. parvulus* 2.6, the studies of bacteria–zebrafish interactions performed in this work support the potential utilization of this bacterium as a probiotic in aquaculture.

Intensive fish farming causes animals to be subjected to stress conditions that weaken their immune system, increasing susceptibility to pathogens and thus promoting the emergence of diseases. Therefore, to prevent and control infections, the immunostimulants are currently utilized as feed additives, as an alternative approach to control these problems (Ringø et al., 2012). The most widely used is the β-glucan with (1,3)-linkages from *Saccharomyces cerevisiae* yeast cell wall (Immunogen® and A-Max are commercialized products) containing β-glucans and mannanoligosaccharides (Yar Ahmadi et al., 2014; Akrami et al., 2015). These products have a positive effect on innate immune parameters, growth, feed efficiency, and resistance against *Aeromonas hydrophila* of rainbow trout. In this work, the exposure of gnotobiotic zebrafish larvae to the *P. parvulus* 2.6 β-glucan produced the inhibition of gene expression of two pro-inflammatory cytokines, TNFα and IL8. In addition, the protein adaptor MyD88, which mediates activation of pro-inflammatory cytokines via NF-κB, was inhibited. TNFα is one of the most important cytokines involved in inflammation responses (Zelová and Hošek, 2013). On the other hand, IL8 (also called CXCL8) is a member of the chemokines family, which facilitates in immune cells (mainly neutrophils) their migration, accumulation, and activity at the inflammation sites (de Oliveira et al., 2013). The neutrophil recruitment is mediated by two G-protein coupled receptors, CXCR1 and CXCR2 (Oehlers et al., 2010), and the use of an induced inflammation model in the *Tg*(*mpx:GFP*) zebrafish line has allowed us to study the behavior of the neutrophils in the presence of the *P. parvulus* 2.6 β-glucan. The results showed that the recruitment and proliferation of the neutrophils in the larvae exposed to the β-glucan were inhibited. Thus, these results support that the 2-substituted (1,3)-β-D-glucan specifically affects the signaling pathway of IL8 producing an anti-inflammatory response, corroborating the anti-inflammatory activity of this polymer on human macrophages (Notararigo et al., 2014). However, Lindström et al. (2012) showed that the purified *P. parvulus* 2.6 β-glucan did not produce any anti-inflammatory response in an *in vivo* mice model, and this discrepancy could be due to differences in the procedures for isolation and purification of the EPS. Nevertheless, in the mice model was detected a higher pro-inflammatory response to 2.6NR than to 2.6 strains (Lindström et al., 2012). Thus, this last result correlates with our previous findings showing differential *in vitro* immunomodulation of human macrophages by these two strains (Fernández de Palencia et al., 2009). In addition, they support that the natural presence of the β-glucan attached to the cell wall of 2.6 strain counteracts the pro-inflammatory effect of *P. parvulus*.

Finally, the current problems in aquaculture indicate that a combination of polysaccharides with immunomodulatory capacity and a probiotic bacterium could be of interest for the improvement of fish production, since they can be utilized to generate functional symbiotic feed for use in fish farms (Huynh et al., 2017). In this context, the study presented here supports the use of *P. parvulus* 2.6 as a probiotic producing *in situ* its β-glucan for the elaboration of fish feed with potential anti-inflammatory and anti-pathogenic properties.

ETHICS STATEMENT

AZTI-Tecnalia has all legal authorization for housing animals, breeding animals, and performing experiments with animals in Spain (animal facility registration number EU-10-BI and REGA code ES489010006105). Dr. Miguel Angel Pardo is responsible of zebrafish facilities and taking part in one of the National Animal Welfare Bodies (Former Ethic Committee), which is accredited for performing animal experiments to the required National and European legislative demands (Council Directive 2010/63/EU), within the project ENVIPHAGE, NEIKER-OEBA-2015-004.

AUTHOR CONTRIBUTIONS

AP-R contributed to all parts of the experimental work and wrote a draft of the manuscript. MP contributed to the design and analysis of the experimental work involving zebrafish models. MM contributed to the design of strategies to develop and analyze the bacterial recombinant strains and corrected the manuscript. PL participated in study conception and data interpretation and generated the final version of the manuscript. All authors read and approved the final manuscript.

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SUPPLEMENTARY MATERIAL

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In situ β-glucan fortification of cereal-based matrices by _Pediococcus parvulus_ 2.6: technological aspects and prebiotic potential. (2017) _International Journal of Molecular Science_ 18, 1588

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CHAPTER 6

Resumen

El uso de bacterias ácido lácticas (BAL) probióticas para la fermentación de alimentos, o simplemente su adición a matrices alimentarias para el desarrollo de nuevos alimentos funcionales, actualmente es un proceso de gran interés. A su vez, los exopolisacáridos (EPS) de BAL son también de gran interés para la industria alimentaria por su capacidad para mejorar las propiedades organolépticas de los alimentos. Por otro lado, en países del norte de Europa y de Asia el desarrollo de alimentos fermentados de matrices no lácteas tiene una gran aceptación por los consumidores. En este contexto, el objetivo de este trabajo fue realizar un estudio comparativo de la capacidad de *P. parvulus* 2.6 y 2.6NR, cepas productora y no productora de (1,3)-β-D-glucano ramificado en posición *O*-2 (β-glucano) para fermentar matrices basadas en cereales. Se generaron 3 matrices liquidas diferentes, utilizando harina (18% w/v) de avena, de arroz, y de cebada. La capacidad fermentativa de las cepas de *P. parvulus* se evaluó durante 64 horas monitorizando la viabilidad bacteriana y el decrecimiento del pH de las matrices. Los resultados mostraron, que las cepas eran incapaces de fermentar la matriz de cebada, por lo que fue descartada. La viabilidad de ambas estirpes fue mayor en la matriz de avena. Sin embargo, el pH disminuyó más rápidamente en la matriz de arroz, alcanzando un pH de 4.0 a las 24 h de fermentación, mientas que en la matriz de avena se necesitaron 40 h para alcanzar dicho pH. La producción del β-glucano de *P. parvulus* en las matrices se valoró mediante el método inmunológico desarrollado en el Capítulo 1 de esta tesis. Las matrices no interfirieron en su cuantificación, ni siquiera los β-glucanos de origen vegetal y presentes en la matriz de avena, demostrando la alta especificidad del método inmunológico. La mayor viabilidad de *P. parvulus* 2.6 en la matriz de avena se tradujo en una mayor producción de β-glucano, 340 mg L^{-1} a las 40 h de fermentación, frente a los 130 mg L^{-1} producidos en la matriz de arroz. Esta diferencia en los niveles de EPS estuvo correlacionada con la viscosidad de los productos fermentados con la cepa 2.6, siendo superior en la matriz de avena que en la matriz de arroz. Además, como era de esperar las matrices fueron más viscosas que aquellas fermentadas con la cepa 2.6NR. Así, los resultados obtenidos mostraron que las matrices de avena y arroz pueden ser utilizadas para la elaboración de nuevos alimentos funcionales fermentados por *P. parvulus* 2.6.

Como ya se ha descrito en el Capítulo 5 de esta tesis y en Notararigo y cols. (2014), el β-glucano producido por *P. parvulus* 2.6 es capaz de modular el sistema inmune y

promover un efecto antiinflamatorio. Además, resultados previos de nuestro laboratorio (Russo et al., 2012) indican que este EPS podría ser fermentado por otras bacterias de la microbiota ejerciendo un efecto prebiótico. Así, podría utilizarse como ingrediente alimentario, pero debido a que los niveles de producción son bajos la mejor opción es su producción *in situ* por la estirpe 2.6 en las matrices. Por esta razón, en este trabajo también se analizaron las matrices fermentadas por las estirpes de *P. parvulus* como vehículos para la administración de otras bacterias probióticas, comparando las matrices enriquecidas en β-glucano bacteriano con las que no lo estaban. Primero, las matrices fermentadas se esterilizaron a 80 ºC durante 20 min, se inocularon con *Lactobacillus plantarum* WCFS1, y se analizó su viabilidad durante: (i) una fermentación de 120 h a 37 ºC; (ii) un proceso de almacenamiento en frio a 4 ºC durante 21 días y (iii) un proceso de estrés gastrointestinal simulado.

Durante la fermentación a 37 ºC en la matriz de avena, la viabilidad incrementó durante las primeras 48 h, siendo el doble en la matriz enriquecida con el β-glucano bacteriano y posteriormente se mantuvo estable. En el caso de la matriz de arroz, a partir de las 48 h la viabilidad disminuyó drásticamente (posiblemente debido al menor pH), siendo dicha disminución 10 veces superior en la matriz no enriquecida, por lo que presumiblemente el β-glucano debe ejercer un efecto protector sobre *L. plantarum* WCFS1 en esta matriz. El almacenamiento a 4 ºC durante 21 días de los productos fermentados no afectó la viabilidad de la cepa WCFS1. Por último, las matrices que contenían *L. plantarum* fueron sometidas secuencialmente a un estrés oral, gástrico e intestinal. En general en todas las matrices, la viabilidad de *L. plantarum* disminuyo al ser sometido a los estreses oral y gástrico, mientras que al someter la bacteria al estrés intestinal provocó una recuperación de sus niveles de viabilidad. En la matriz de arroz no se encontraron diferencias entre la matrices enriquecidas o no con el β-glucano bacteriano, mientras que en la matriz de avena durante el estrés intestinal la recuperación de la viabilidad fue significativamente mayor en la matriz enriquecida, lo que indica que la presencia del β-glucano de *P. parvulus* 2.6 confiere un efecto protector a *L. plantarum* WCFS1 en esta matriz.

Mi aportación a este trabajo

Para lograr la consecución de este trabajo científico, mi aportación fue la siguiente. Contribuí a la realización de todo el trabajo experimental. También, realicé el borrador de los apartados de materiales y métodos y resultados del manuscrito, así como las figuras y tablas que contiene.

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In Situ β-Glucan Fortification of Cereal-Based Matrices by *Pediococcus parvulus* 2.6: Technological Aspects and Prebiotic Potential

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Abstract: Bacterial exopolysaccharides produced by lactic acid bacteria are of increasing interest in the food industry, since they might enhance the technological and functional properties of some edible matrices. In this work, *Pediococcus parvulus* 2.6, which produces an *O*2-substituted (1,3)-β-D-glucan exopolysaccharide only synthesised by bacteria, was proposed as a starter culture for the production of three cereal-based fermented foods. The obtained fermented matrices were naturally bio-fortified in microbial β-glucans, and used to investigate the prebiotic potential of the bacterial exopolysaccharide by analysing the impact on the survival of a probiotic *Lactobacillus plantarum* strain under starvation and gastrointestinal simulated conditions. All of the assays were performed by using as control of the *P. parvulus* 2.6's performance, the isogenic β-glucan non-producing 2.6NR strain. Our results showed a differential capability of *P. parvulus* to ferment the cereal flours. During the fermentation step, the β-glucans produced were specifically quantified and their concentration correlated with an increased viscosity of the products. The survival of the model probiotic *L. plantarum* WCFS1 was improved by the presence of the bacterial β-glucans in oat and rice fermented foods under starvation conditions. The probiotic bacteria showed a significantly higher viability when submitted to a simulated intestinal stress in the oat matrix fermented by the 2.6 strain. Therefore, the cereal flours were a suitable substrate for in situ bio-fortification with the bacterial β-glucan, and these matrices could be used as carriers to enhance the beneficial properties of probiotic bacteria.

Keywords: *Pediococcus parvulus*; exopolysaccharides; β-glucans; functional foods; bio-fortification

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1. Introduction

Bacterial exopolysaccharides (EPS) are extracellular polymers which can be tightly adhered (i.e., capsular), loosely associated to the microbial cell surface, or released into the environment [1–4]. Lactic acid bacteria (LAB) are a heterogeneous group of Gram-positive prokaryotes characterised by a long history of safe use by humans, and are of outstanding relevance for the production of fermented food and beverages [5,6]. Several LAB strains are able to produce EPS, either heteropolysaccharides or homopolysaccharides, mainly including β-D-glucans, α-D-glucans, levans, and β-D-galactans [2]. Based on their functional and technological properties, bacterial EPS are of interest for new developments in food, biomedical, and pharmaceutical applications [7,8]. It is

CHAPTER 7

7. Unpublished results

7.1. Effect of different abiotic stresses on the growth of *P. parvulus* strains

The subject of this thesis has been the β-glucan-producing *P. parvulus* 2.6 due to its potential as a probiotic bacterium and that it could be used for the development of various functional fermented foods enriched in 2-substituted (1,3)-β-glucan. I have shown in chapter 6 of this thesis the suitability of the bacterium for the production of cereal-based products enriched in this bacterial β-glucan.

During the production of fermented food, and depending on the matrices used, the starter bacteria could be exposed to different abiotic stresses. The production of dairy products, such as yogurt, is characterised by the acidification of the milk to values even lower than pH 4. Osmosis is also an important factor in the preparation of some fermented foods such as cheese or meat, and the starter bacteria should tolerate a certain concentration of salinity. Moreover, during the fermentation processes, production of oxidative agents could occur and could affect the metabolism and growth of the bacteria. In addition, the manufacture of fermented beverages such as wine, beer or cider entails the production of ethanol that can also affect the bacterial growth. Thus, in this work I subjected the β-glucan-producing *P. parvulus* 2.6 and its isogenic non-producing strain 2.6NR to the above technological stresses to evaluate their resistance.

To determine the tolerance to the different stresses, the bacteria were grown in MRS (Pronadisa) media and the optical density at 600 nm (OD_{600nm}) of the cultures was monitored. To analyse the bacterial response to the acidic stress, the growth medium was adjusted to pH 6.2, 4.5 or 3.5. To evaluate the response of the pedioccoci to the other stresses the medium was supplemented as follows. For the osmotic constraint, NaCl was added at either 2% or 4%. For the oxidative stress, concentrations of *N*,*N*,*N*´,*N*´-tetramethylazo-dicarboxamide (diamide) from 0.5 mM to 2 mM were tested. Finally, for the ethanol stress, concentrations of the alcohol from 2 % to 8 % were tested.

Our results revealed that the initial pH of the MRS media affected the growth of both pediococcal strains, which showed the same pattern for each condition tested (Fig. 1A). The growth rate for both strains was almost identical, whether grown at pH 6.2 or 4.5 (ranging from 0.048 ± 0.005 to 0.054 ± 0.005). However, upon growth at pH 4.5, both strains reached the stationary phase of growth earlier and at lower OD_{600nm} (0.8) than at pH 6.2 (1.1).

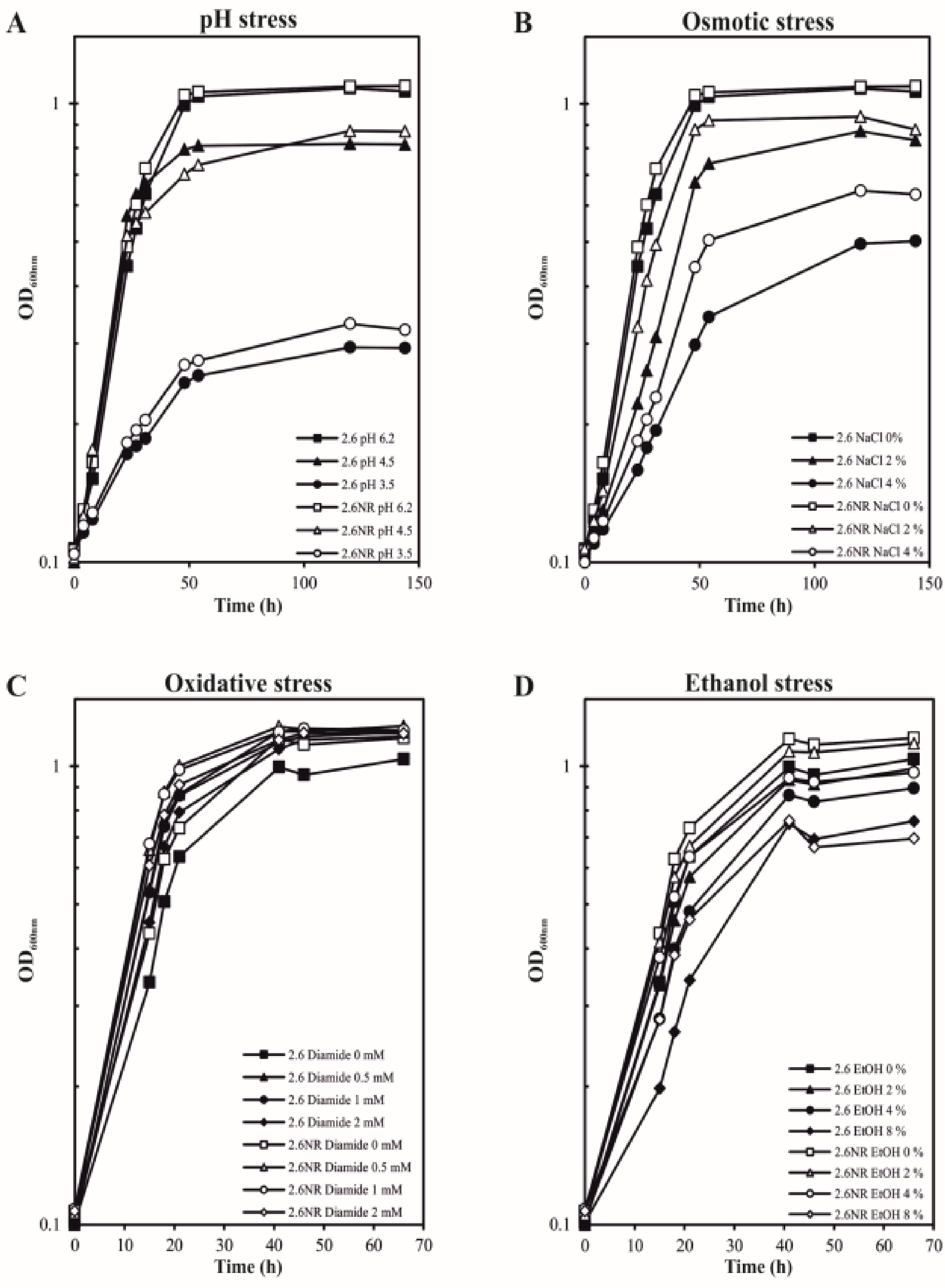


Figure 1. Analysis of tolerance of *P. parvulus* 2.6 (black symbols) and 2.6NR (white symbols) strains to abiotic stresses: pH (**A**), osmosis (**B**), oxidation (**C**) and ethanol (**D**). The bacterial growth was monitored by measurement of the OD_{600nm}. The symbols are described in each graphic.

In addition, in MRS pH 3.5 the growth of both strains was drastically reduced. At the middle of the exponential phase, their growth rate (0.014 ± 0.002) was about 4-fold lower than that in medium at pH 6.2. Moreover, the final OD_{600nm} in MRS pH 3.5 was very low only about 0.3. The final pH of all the cultures was determined at the end of the growth and the values were around pH 3.3-3.4 for the cultures with initial pH 4.5 or 3.5, and values of more than pH 4 for the cultures with initial pH 6.2. These results indicate that *P. parvulus* strains are not able to acidify the medium below pH 3.3 and at this point the growth is seriously affected.

The results obtained after applying salinity stress showed that the pediococcal strains could withstand a NaCl concentration of even 4% (Fig. 1B). However, the exposure resulted in a latent period more pronounced for 2.6 than for 2.6NR. The cultures grown in the presence of 2% NaCl showed a similar growth rate (0.047 ± 0.004) in the middle of the exponential phase as that of those not subjected to osmotic stress, achieving a final OD_{600nm} of 0.9. However, when the bacteria were exposed to a salt concentration of 4% the growth rate decreased about 2-fold (0.025 ± 0.001), and the OD_{600nm} achieved for the cultures only reached values of 0.5 or 0.6 for 2.6 or 2.6NR, respectively (Fig. 1B). Thus, the overall results revealed that *P. parvulus* 2.6NR is more resistant than 2.6 strain to the osmotic stress.

Diamide is a thiol-specific oxidant that generates reactive oxygen species. The exposure to this compound had no negative effect on the growth of either pediococcal strain at any of the concentrations tested (Fig. 1C). Moreover, a slight growth improvement was observed in the presence of diamide.

Finally, the bacteria were subjected to an ethanol stress. During the production of fermented alcoholic beverages, bacteria must withstand different alcoholic concentrations; besides *P. parvulus* 2.6 was isolated from natural cider which can achieve ethanol concentration of 5-6%. Consequently, and as expected, the results showed that both strains could grow even in the presence of 8% ethanol with only 10% decrease in the growth rate (from 0.097 ± 0.011 to 0.086 ± 0.006) (Fig. 1D). In addition, an increase of viscosity in the *P. parvulus* 2.6 cultures was observed in the presence of ethanol. This fact could mean an increase in β-glucan production, therefore we analysed the β-glucan levels as well as the cell viability in cultures subjected to ethanol stress. For this study, the bacteria (10^8 cfu mL^{-1}) were subjected to 4%, 8% and 12% ethanol. Both strains, 2.6 and 2.6NR, grown in MRS without ethanol reached the stationary phase at OD_{600nm} of 1.1 (Fig. 2A) with the same growth rate (0.068), after 48 h of growth. Regarding to viable cells, the cultures reached values of 3.5 × 10^9 cfu mL^{-1} or 5.4 × 10^9 cfu mL^{-1} for 2.6 or 2.6NR strains, respectively (Fig. 2B). Then, after 72 h of incubation, the cell viability decreased to 2.7 ×10^9 cfu mL^{-1} and 4.2 × 10^9 cfu mL^{-1} for 2.6 and 2.6NR, respectively. These results were taken as a control to analyse the behaviour of both strains in the presence of ethanol. Thus, these viability values of both strains, at 48 h and 72 h, were taken as the 100% values.

The presence of 4% ethanol did not substantially affect the bacterial growth. Both strains achieved the stationary phase after 48 h with an OD_{600nm} of approximately 1.0 and a growth rate of 0.062. The viable bacteria detected at this point, were 2.5 ×10^9 cfu mL^{-1} for 2.6 and 3.4 × 10^9 cfu mL^{-1} for 2.6NR, corresponding to a decrease in cell viability of 30% and 40% compared to the controls. Furthermore, after 72 h of growth the survival of 2.6 remained stable, while the cell viability of 2.6NR decreased 60%.

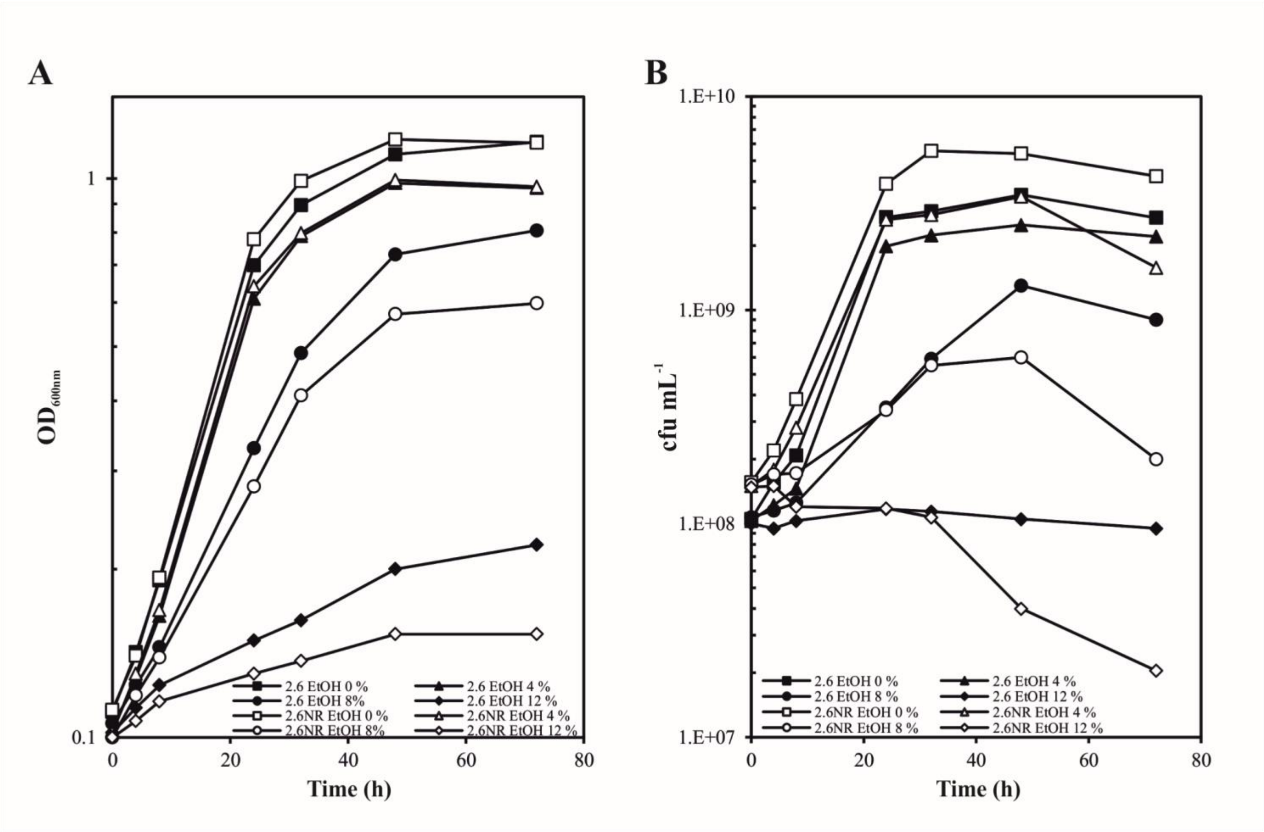


Figure 2. Analysis of the ethanol effect in growth and viability of 2.6 (black symbols) and 2.6NR (white symbols) strains. The bacterial growth was detected by measuring the OD_{600nm} (A) and the viability was measured by platting determination of the cfu mL^{-1} (B). All the symbols are described in each graphic.

In the presence of 8% ethanol, both strains had a growth rate of 0.04, however the OD_{600nm} reached after 48 h of growth was higher for 2.6 (0.73) than for 2.6NR (0.57). In addition, viable cells were different for both strains at this point, achieving 1.3×10^9 cfu mL^{-1} for 2.6 and 6×10^8 cfu mL^{-1} for 2.6NR, and these values showed a reduction of cell survival of 60% and 90% respectively, compared to the controls. As happened in the presence of 4% ethanol, 2.6 viability was stable after 72 h of growth, while 2.6NR viability was reduced in 95%.

Finally, the results showed that 12% ethanol was a limiting dose for the pediococcal growth, since cultures of 2.6 and 2.6NR strains only reached an OD_{600nm} of 0.22 and 0.15, respectively, which growth rates of 0.025 and 0.019. In addition, the study of the viable cells showed that this ethanol concentration impaired bacterial growth. Moreover, 2.6 retained its viability (about 1.0×10^8 cfu mL^{-1}) during all the treatment period. By contrast, 2.6NR maintained its viability only during the first 24 h of treatment, and then its survival was reduced 73% and 87% after 48 h and 72 h of treatment, from 1.5×10^8 cfu mL^{-1} at the beginning of growth to 4.0×10^7 cfu mL^{-1} and 2.0×10^7 cfu mL^{-1}, respectively.

The analysis of the 2-substituted (1,3)-β-glucan production by *P. parvulus* 2.6 showed that in the presence of ethanol the bacterium increased its production of EPS (Table 1). There were no significant differences in the total concentration detected. However, when production per cell was calculated (mg of β-glucan/cfu), these results revealed that the production increased when the ethanol concentration in the medium increased. The production of the β-glucan was 3-fold higher in the presence of 8% ethanol and up to 10-fold higher in the presence of 12% ethanol. These results suggest a protective effect of this β-glucan against ethanol stress, and ethanol is likely to trigger activation of expression of genes involved in the biopolymer production, but to prove this hypothesis requires further work.

Table 1. β-glucan production by *P. parvulus* 2.6 strain in MRS cultures after 48 h of growth in the presence of ethanol

Ethanol dose	**β-glucan (mg L^{-1})**	**Viability (cfu mL^{-1})**	**Ratio (mg of β-glucan/cfu)**
EtOH 0 %	467.7 ± 56.3	3.46×10^9	0.14
EtOH 4 %	470.1 ± 52.9	2.50×10^9	0.19
EtOH 8 %	529.8 ± 60.4	1.30×10^9	0.41
EtOH 12 %	164.5 ± 29.0	1.05×10^8	1.57

The overall results showed that the technological properties of *P. parvulus* 2.6 are adequate for its inclusion in the manufacture of fermented foods. This perhaps is self-evident in the production of alcoholic beverages since strains of *P. parvulus* have been isolated from cider and wine. It also offers the possibility of use in other foods, in which the salinity does not exceed 4%, acidity would not be lower than pH 3.5 and even if oxidative agents were to be produced. Thus, this bacterium could be used for the production of yogurt-like foods based on cereals, as shown in Chapter 6 of this thesis.

7.2. Improvement of sorbitol uptake by *P. parvulus* 2.6

As already mentioned in Chapter 4 of this thesis, the efficiency of 2.6 strain to metabolise sorbitol was very low, compared with its utilisation of other carbon sources, e. g. glucose. In this work the bacterium was grown in a non-commercial MRS made by components without carbon source and supplemented with 30 mM sorbitol and without adjusting the pH (pH of the medium 6.5). Under these conditions, the growth of *P. parvulus* 2.6 was very poor taking more than 10 days to achieve an OD_{600nm} of 3.0 (see supplementary material Fig. S1 of Chapter 4). Therefore, this study was performed with the aim to identify the optimal growth conditions to improve sorbitol metabolism. The influence of the pH of the medium and the usage of other carbon sources at limiting concentrations (15 mM) together with sorbitol was tested and the results are depicted in Figure 3. The growth of the 2.6 strain in MRS pH 6.5 in the presence of the three carbon source glucose (MRSG), fructose (MRSF) and maltose (MRSM) was quite different (Fig. 3A and 3B), having a growth rate in the exponential phase of 0.2, 0.3 and 0.1, respectively. The graphs depict the late exponential and the stationary phases to assess the influence of the sorbitol on the bacterial metabolism. However, no differences were observed between cultures in the presence (S) or absence of sorbitol, achieving a final OD_{600nm} of 2.2 in MRSG and MRSGS and 3.1 in MRSF and MRSFS. The growth of 2.6 in the presence of maltose was slightly improved when the medium contained sorbitol, achieving a final OD_{600nm} of 2.8 MRSMS *versus* 2.0 in MRSM. This behaviour of 2.6 strain during the co-metabolism of maltose and sorbitol was also detected at pH 5.0 and 4.0 (Fig. 3D and 3F), but under these conditions the growth rates were in all cases less than 0.04. These results obtained in the presence of maltose, made us discard this carbon source to perform further studies of co-metabolism with sorbitol.

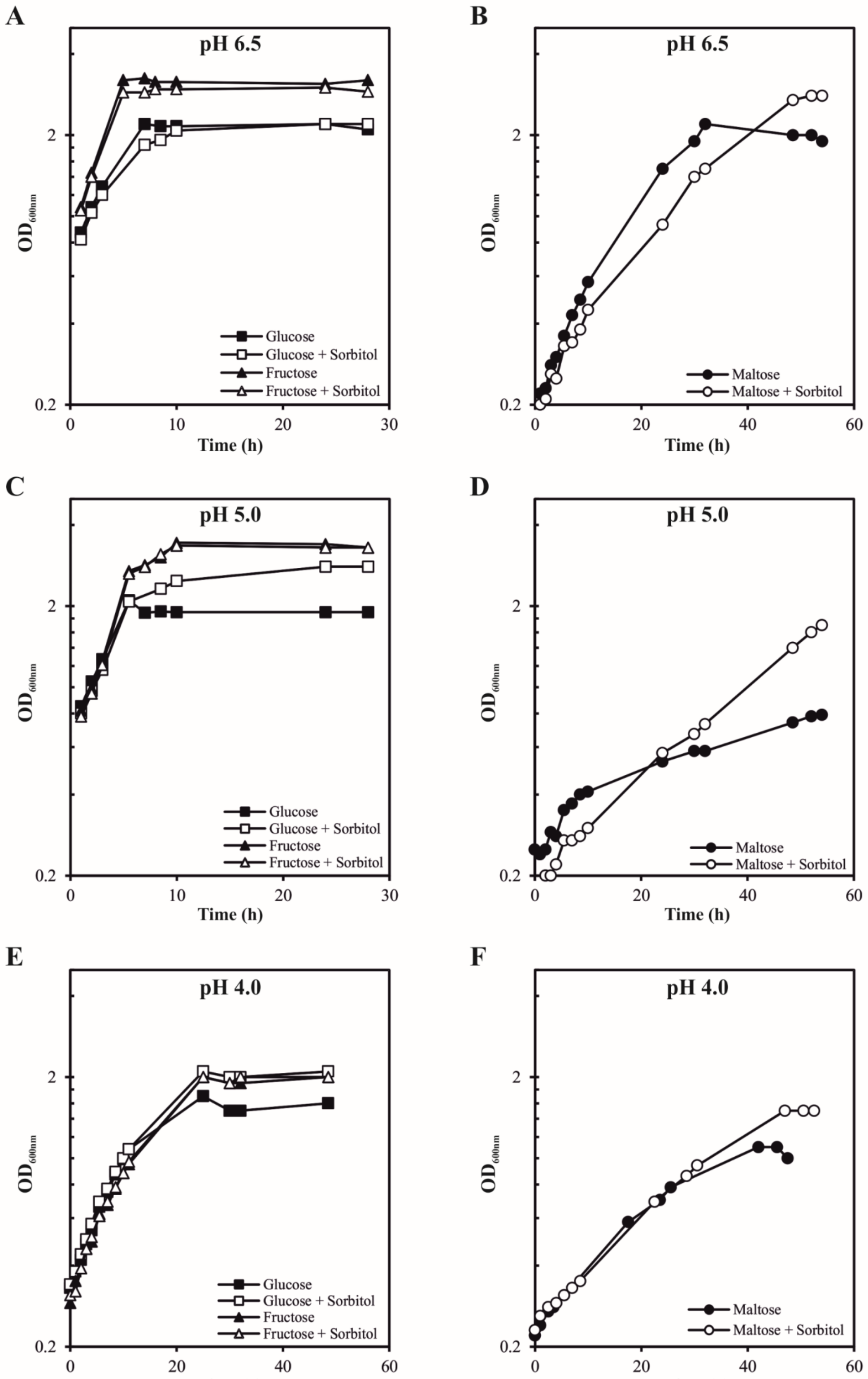


Figure 3. Analysis of the influence of pH and additional carbon sources on co-metabolism with sorbitol in *P. parvulus* 2.6. The pH was evaluated at 6.5 (**A** and **B**), 5.0 (**C** and **D**) and 4.0 (**E** and **F**). MRS was supplemented with 15 mM glucose (squares), 15 mM fructose (triangles) or 15 mM maltose (circles) or adding 30 mM sorbitol (white symbols). The bacterial growth was measured by monitoring the OD$_{600nm}$.

The growth of 2.6 in MRSG and MRSF at pH 5.0 had similar growth rates of 0.20 and 0.22 respectively. In these conditions, the 2.6 cultures in MRSF and MRSFS reached the same final OD_{600nm} of 3.3 (Fig. 3C). However, the 2.6 growth in MRSGS reached a higher final OD_{600nm} that in MRSG (2.8 *versus* 1.9). These behaviours were maintained in medium adjusted to pH 4.0, but under these conditions the growth was reduced (Fig. 3E). The growth of 2.6 in MRSG as well as in MRSF at pH 4 showed growth rates below 0.1, and achieved final OD_{600nm} of 1.6 and 2.0, respectively. Under these conditions, the presence of sorbitol also slightly improved the growth in MRSG, up to a final OD_{600nm} of 2.0. The overall analysis of the results, made us discard also the MRSFS and a pH medium of 4.0 to improve the sorbitol metabolism. For this reason, further analyses were performed in MRSGS. In addition, the concentration of glucose was reduced to 10 mM to appreciate better the influence of the sorbitol in growth. Moreover, the influence of the aeration was tested incubating the cultures under aerobic conditions (shaking at 180 rpm). The results are depicted in Figure 4. In all conditions, the cultures had similar exponential phases, with growth rates ranging from 0.17 to 0.19.

After 9 h of incubation the 2.6 cultures grown in MRSG entered into the stationary phase, and the final OD_{600nm} reached for these cultures was around 1.8. However, the 2.6 cultures grown in MRSGS had different behaviours depending on growth conditions. The pH as well as the aeration of the cultures affected the growth of 2.6 in the presence of sorbitol. Thus, at pH 6.5 and under static conditions the use of sorbitol was not very efficient, reaching the cultures only an OD_{600nm} of 2.0. When aeration was provided, the final OD_{600nm} of 2.6 strain was 2.25. Also, when the MRSGS was adjusted to pH 5.0, without aeration, the final OD_{600nm} was 2.7, and when the culture was aerated the best yield was achieved, with a final OD_{600nm} of 3.8.

Thus, the better conditions for sorbitol uptake and metabolism by *P. parvulus* 2.6 were growth of the bacterium in MRS supplemented with 10 mM glucose plus 30 mM sorbitol at pH 5 and under aerobic condition (Fig. 4D). Consequently, these preliminary experiments defined the conditions for the characterization of the sorbitol metabolism performed in Chapter 4 of this thesis.

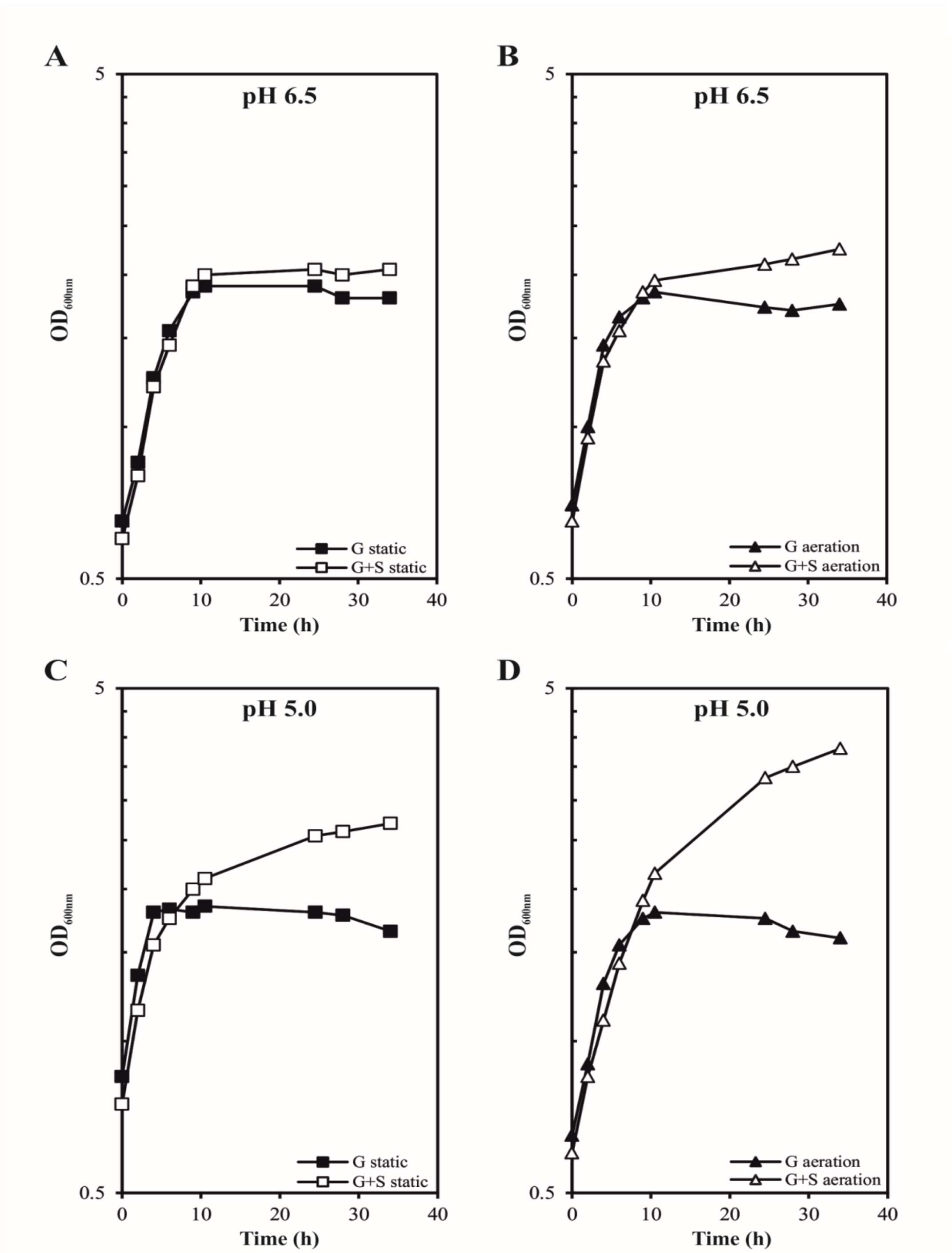


Figure 4. Study of glucose and sorbitol co-metabolism by *P. parvulus* 2.6. The influence of the pH was tested at 6.5 (**A** and **B**) and 5.0 (**C** and **D**). Also, the incubation was assayed under either static (squares) (**A** and **C**) or aerobic (triangles) (**B** and **D**) conditions. MRS was supplemented with 10 mM of glucose (black symbols) or with 10 mM glucose plus 30 mM sorbitol (white symbols).

7.3. Analysis of the regulation of the expression of the *gut* operon in *L. plantarum* WCFS1

The *gut* operon for sorbitol utilization detected and characterised in *P. parvulus* 2.6 carries the *gutR* and *gutM* genes, which encode the GutR and GutM regulatory proteins. In Chapter 4 of this thesis, a functional study of expression of both proteins and the promoter region P*gut* of the 2.6 was carried out, using two heterologous systems: *L. plantarum* 90 and *L. casei* BL23, which had *gut* operons and promoter regions, respectively with high and low homology to that of *P. parvulus* 2.6.

However initially, during the development of this PhD work, we had chosen to perform the study the *L. plantarum* WCFS1 that had a *gut* operon equal to that of *L. plantarum* 90, instead of the 90 strain. The WCFS1 strain is the prototype of *L. plantarum* strains and for this reason has been better characterised than the 90 strain and more information was available. Therefore, it was deemed to be a better choice for the study. The gene expression study was carried out using the pRCR16-pRCR19 plasmids, which contain regions of the sorbitol utilization genetic determinants. They were constructed as described in Chapter 4, using the promoter-probe vector pRCR (Mohedano et al., 2015a), which carries the reporter gene *mrfp* that encodes the monomeric red fluorescence protein mCherry. Thus, WCFS1 was transformed independently with the 4 plasmids: pRCR16 (4.2 kbp, carrying the promoter region P*gut*), pRCR17 (6.1 kbp, carrying the transcriptional fusion P*gut-gutR*), pRCR18 (4.8 kbp, carrying the transcriptional fusion P*gut-gutM*) and pRCR19 (6.6 kbp, carrying the transcriptional fusion P*gut-gutRM*). Recombinant strains were successfully obtained in all 4 cases.

The complete genome sequence of *L .plantarum* WCFS1 has been determined and revealed that this bacterium carries 3 natural plasmids: two small that replicate via the rolling circle mechanism, pWCFS101 (1.9 kbp) and pWCFS102 (2.4 kbp); and one large that replicates via the theta type mechanism, pWCFS103 (36.1 kbp) (Kleerebezem et al., 2003; van Kranenburg et al., 2005). Therefore, the plasmid profiles of the WCFS1 and its recombinant derivative strains were analysed in an 0.7% agarose gel (Fig. 5). The analysis revealed the expected pRCR derivatives in the corresponding bacteria. However, the results also revealed that all the recombinant strains had lost the pWCFS102 plasmid. Analysis of the DNA sequences of the replicons of pWCFS102 and pRCR revealed that both belong to the pMV158 family (Lorenzo-Díaz et al., 2014). This fact could provoke incompatibility between pWCFS102 and the pRCR derivatives.

We were able to establish the pRCR derivatives in *L. plantarum* WCFS1, due to the selective pressure imposed on the strain by plating in media containing chloramphenicol (since the plasmids encode a chloramphenicol acetyl transferase). Thereby, this selection and further growth of the transformants in the presence of the antibiotic presumably resulted in the loss of pWCFS102. This event led us to discontinue the usage of *L. plantarum* WCFS1 for studies of sorbitol metabolism and regulation and to include as heterologous system *L. plantarum* 90, which has not presented problems of plasmid incompatibility to perform the experiments described in Chapter 4.

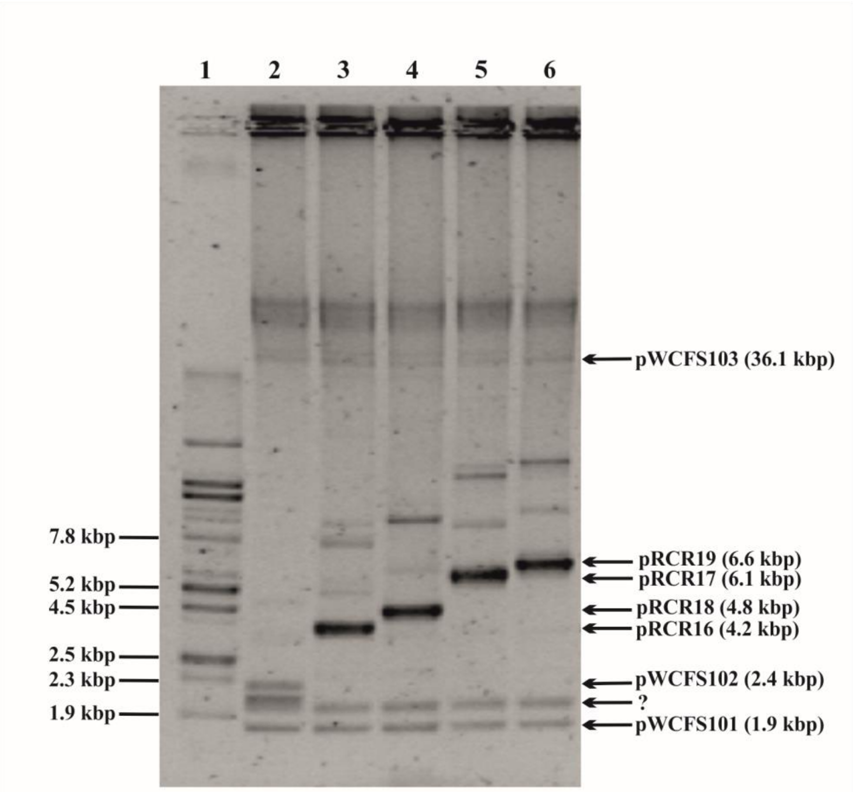


Figure 5. Plasmid analysis of *L. plantarum* WCFS1 carrying pRCR derivatives in a 0.8% agarose gel. Plasmidic DNA preparation of, Lines: 1, *E. coli* V517, a multi-plasmid strain used as standard (plasmid sizes are depicted); 2, *L. plantarum* WCFS1; 3, WCFS1[pRCR16]; 4, WCFS1pRCR18]; 5, WCFS1[pRCR17]; 6, WCFS1[pRCR19]. The arrows indicate the position of the different plasmids and their sizes. Question-mark indicates an unknown band.

Nevertheless, supplementary regulation studies were performed with WCFS1 recombinant strains, which were not included into the published manuscript (Chapter 4 of this thesis). First, the induction of P*gut* from 2.6 by the regulatory proteins of WCFS1 strain in the presence of sorbitol were analysed (Fig. 6). To this aim, *L. plantarum* WCFS1[pRCR16] after growth in MRS supplemented with 1% glucose, was transferred to MRS fresh medium supplemented with either 1% sorbitol or 1% glucose, and the OD_{600nm} as well as the fluorescence emitted by the cells were monitoring. The results revealed that the promoter P*gut* in the presence of glucose was not activated, and the presence of sorbitol as inductor was needed for its expression (Fig. 6A and 6B). Thus, in the presence of sorbitol the cultures started to emit fluorescence, progressively with time, and at the end of the experiment (6 h), almost all the cells in the culture became fluorescent (Fig. 6A).

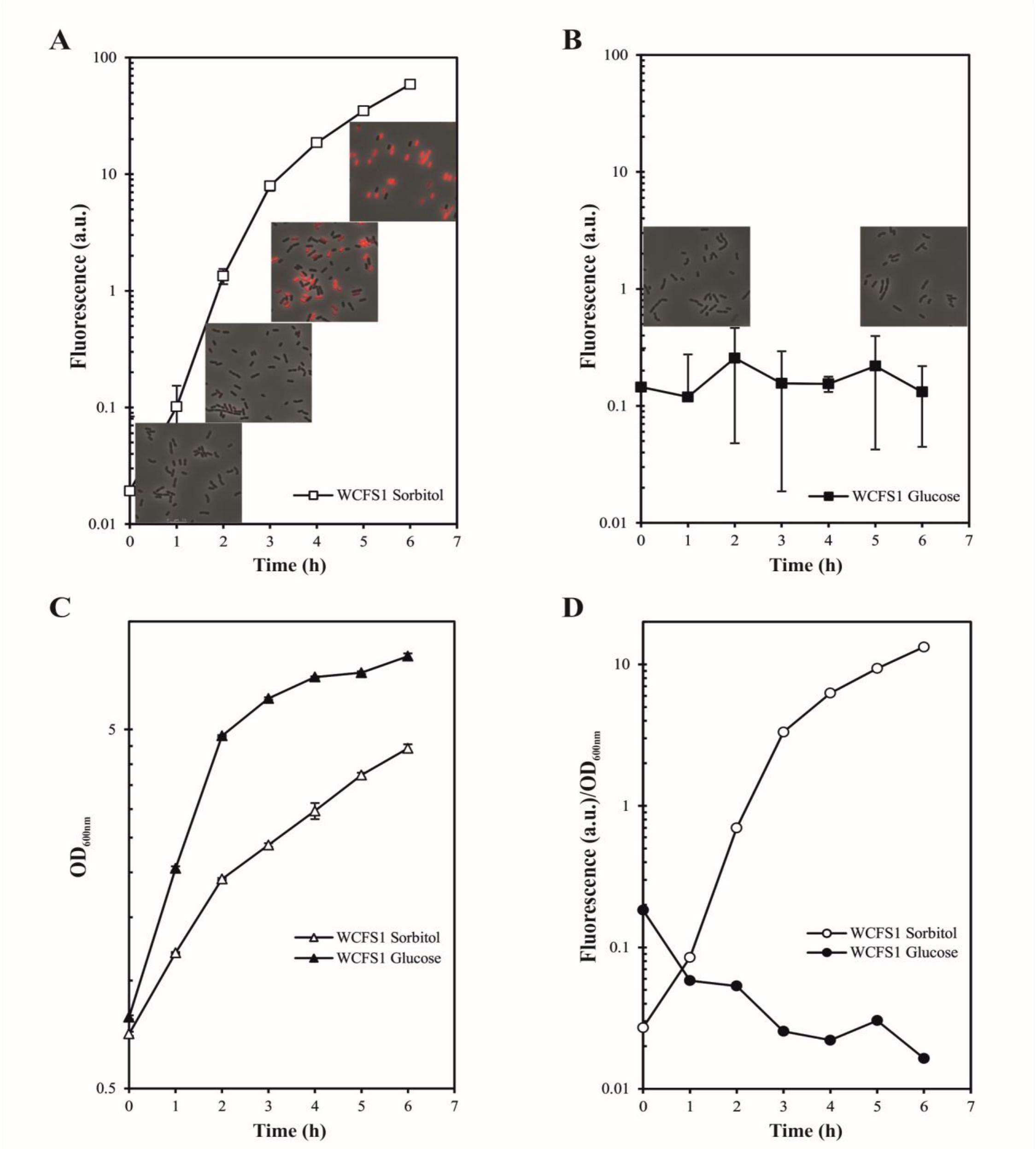


Figure 6. Trans-complementation of the sorbitol utilization regulatory machinery in *L. plantarum* WCFS1[pRCR16]. The Bacterium was grown in MRS supplemented with 1% glucose, and at time 0 was transferred to fresh MRS supplemented with 1% sorbitol (white symbols) or with 1% glucose (black symbols). Fluorescence (**A** and **B**) and OD_{600nm} (**C**) of the cultures were monitored every hour. Fluorescence/OD_{600nm} ratio of the cultures was determined (**D**). Overlays of images of the cultures taken at time 0 and after 2, 4 and 6 h of incubation by phase contrast and fluorescence microscopy are depicted.

The analysis of the OD_{600nm} showed that the bacterium metabolises glucose better than sorbitol, since the initial growth rates were 0.9 and 0.5, respectively (Fig. 6C). After an incubation of 2 h the culture supplemented with glucose entered into the stationary phase, and the culture supplemented with sorbitol changed its growth rate to 0.21. However, the ratio fluorescence/OD_{600nm} still maintains the exponential increase of fluorescence in the presence of sorbitol (Fig. 6D). These results showed a *trans*-complementation of the *L. plantarum* WCFS1 regulatory proteins on expression driven from the *P. parvulus* P*gut* promoter.

In addition, the analysis of the other constructions transferred to WCFS1 strain was made. In this case, punctual measurements were taken at different phases of the growth (Table 2). The results show that in the presence of the genes that encode for the regulatory proteins of 2.6 strain (pRCR17-19), the expression from the promoter P*gut* in the exponential growth phase is greater than in the strain that carries the pRCR16 plasmid. In this host, it is not possible to elucidate the role played by each of the proteins, since the system is regulated by the host proteins. However, the model proposed in Chapter 4 suggests that GutR (expressed from pRCR17) acts as a transcriptional activator and GutM (expressed from pRCR18) helps to stabilize the regulation process (see more details in Chapter 4), for this reason when both genes are present (expressed from pRCR19) the expression from the promoter is lower. Thus, in the stationary phase of growth, expression from the P*gut* promoter is equal in all constructions except for the strain that overexpresses the *gutM* gene (from pRCR18), where gene expression levels are higher by more than 60%. These results suggest that an overexpression of the protein GutM, without the same levels of the protein GutR produces a deregulation of the system (see further details in chapter 4 of this thesis).

Table 2. Fluorescence emitted by the *L. plantarum* strains carrying the pRCR-derived plasmids. Fluorescence values (a.u.) were normalized with OD_{600nm} values.

Strain	**Exponential phase (5 h)**	**Stationary phase (10 h)**
WCFS1	0.03	0.24
WCFS1[pRCR16]	10.09	24.89
WCFS1[pRCR17]	16.44	24.38
WCFS1[pRCR18]	14.84	38.67
WCFS1[pRCR19]	12.03	22.06

General discussion

P. parvulus 2.6 isolated from Basque Country ropy cider (Fernández et al., 1995) is the prototype of the 2-substituted (1,3)-β-glucan-producing bacteria synthesised by the GTF glycosyltranferase (Werning et al., 2006), and this LAB as well as its HoPS have been the subjects of this thesis. *P. parvulus* 2.6NR (non-ropy) does not produce the polymer, because it was generated by chemical treatment of the 2.6 strain (Fernández et al., 1995), which resulted in the curing of the plasmid carrying the GTF glycosyltranferase coding *gtf* gene (Fernández et al., 1995; Werning et al., 2006). Consequently, throughout this thesis work, we have performed a comparative analysis of the performance of *P. parvulus* 2.6 and its isogenic β-glucan-non-producing 2.6NR strain, which has allowed us to infer the influence of the HoPS and its synthesis on the probiotic, metabolic and technological properties of the 2.6 strain.

1. Genomic characterization of *P. parvulus* 2.6

In this work, the DNA sequence of *P. parvulus* 2.6 genome has been determined (Chapter 2). This allowed us to obtain *in silico* information of the coding sequences (CS), including among others those related to the metabolic capacities that this bacterium possesses (Fig. 1). The genome includes 2175 CS, among them 914 CS belong to a known subsystem category recognised by the RAST database. These 914 CS correspond to 875 genes with homologues in the databank, and 39 hypothetical genes. On the other hand, there are 1261 CS, which do not belong to a known subsystem category, of which 561 are hypothetical genes.

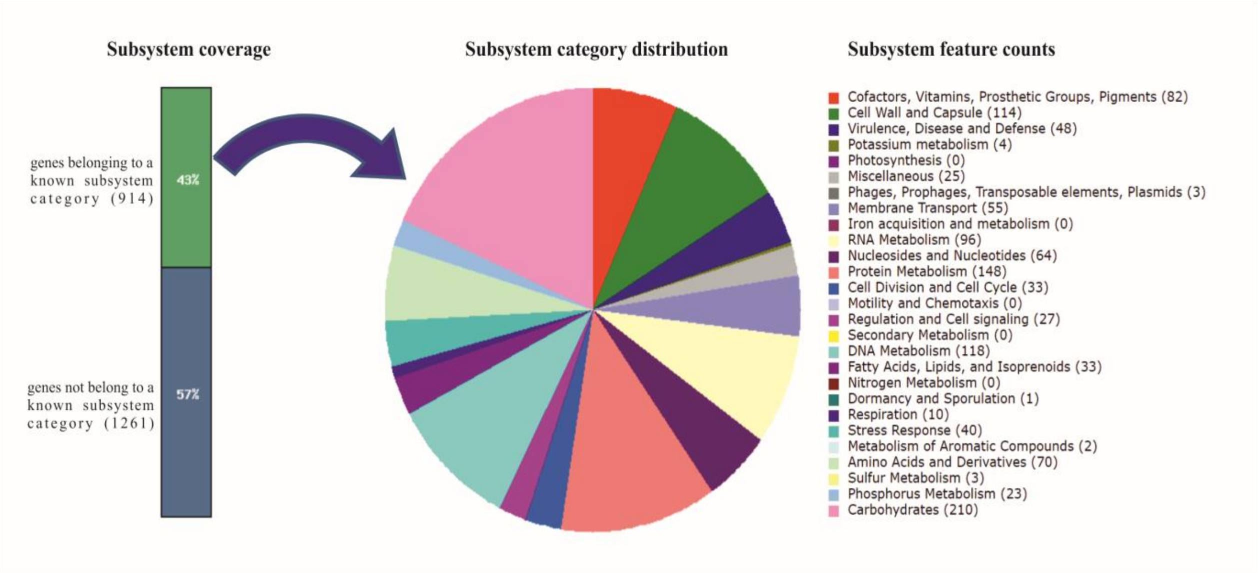


Figure 1. Distribution of coding sequences detected in the genome of *P. parvulus* 2.6. The colours refer to different coverage or categories.

Production of the 2-substituted β-glucan by *Pediococcus* strains isolated from beverages has been extensively described (Dueñas-Chasco et al., 1997; Dols-Lafargue et al., 2008). Furthermore, we have reported this year, for the first instance, a *Pediococcus* (*P. ethanolidurans* CUPV141), which produces in addition to the β-glucan a HePS composed of glucose, galactose, glucosamine, and glycerol-3-phosphate (Llamas-Arriba et al., 2018).

The analysis of the genome allowed the identification of a *cps* cluster of 12 genes involved in HePS synthesis and secretion, homologous to one of those of *L. plantarum* (Fig. 2). Nevertheless, the synthesis of HePS by the 2.6 strain has never been detected and this could be due to a lack of polymer synthesis or production at not detectable levels, because there is a lack or decrease of functionality of one or more of the *cps* genes products e. g. the p-GTF or priming-glycosyltransferase (undecaprenyl-phosphate galactosephosphotransferase product for the 2.6 cluster), which initiates the polymerization.

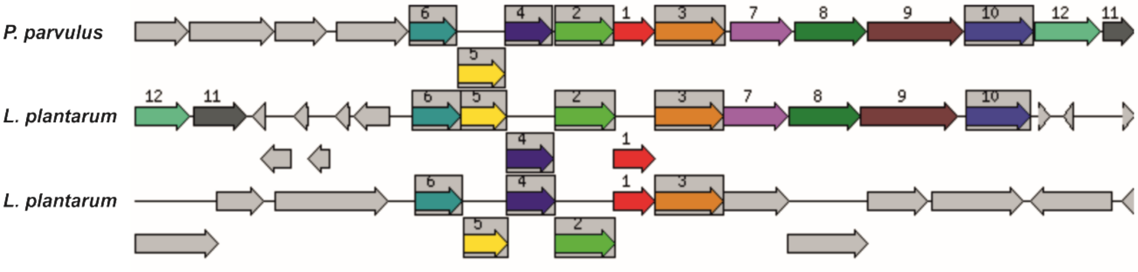


Figure 2. Cluster of genes of *P. parvulus* 2.6 encoding putative proteins responsible for the synthesis and secretion of a HePS. The BLAST alignment with *L. plantarum* WCFS1 clusters was generated with the RAST software and based on the homology of the *p-gtf* gene. Numeric codes for genes encoding: 1, undecaprenyl-phosphate galactosephosphotransferase (EC 2.7.8.6); 2, UDP-glucose 4-epimerase (EC 5.1.3.2); 3, exopolysaccharide biosynthesis glycosyltransferase EpsF (EC 2.4.1.-); 4, manganese-dependent protein-tyrosine phosphatase (EC 3.1.3.48); 5, glycosyltransferase; 6, tyrosine-protein kynase EpsD (EC 2.7.10.2); 7, polysaccharide polymerase; 8, tyrosin-protein kynase transmembrane modulator EpsC; 9, O-antigen flippase Wzx; 10, capsular polysaccharide biosynthesis protein; 11, dTDP-4-dehydrorhamnose reductase (EC 1.1.1.133); 12, d-TDP-glucose 4,6-dehydratase (EC4.2.1.46).

In addition, the analysis of the genome also predicted the existence of 3 independent proteins involved in plasmid replication. These findings are consistent with the results obtained in this work from the analysis of plasmidic DNA preparations of 2.6 in an agarose gel, which revealed that *P. parvulus* 2.6 strain harbours 3 natural plasmids called pPP1, pPP2 and pPP3, whith estimated sizes of 39.1 kbp, 24.5 kbp and 12.7 kbp, respectively (Fig. 4 of Chapter 4 and scheme in Fig. 3A). On the other hand, the 2.6NR strain harbours only two plasmids, the pPP1* and pPP3 plasmids (Fig. 4 of Chapter 4 and scheme in Fig. 3A). The lack of the pPP2 plasmid, which carries the *gtf* gene encoding the enzyme responsible for the synthesis of the β-glucan impairs the HoPS production (Werning et al., 2006; Llamas-Arribas et al., 2018; and Chapter 4). As a consequence, only EPS (Fig. 3B and Fig 3 of Chapter 5) and mucous colonies (Fig. 3C and Fig. 2 of Chapter 5) were detected for the 2.6 strain. The pPP1* plasmid carried by the 2.6NR strain migrates slightly above the pPP1 of 2.6, with an estimated size of 40.0 kbp.

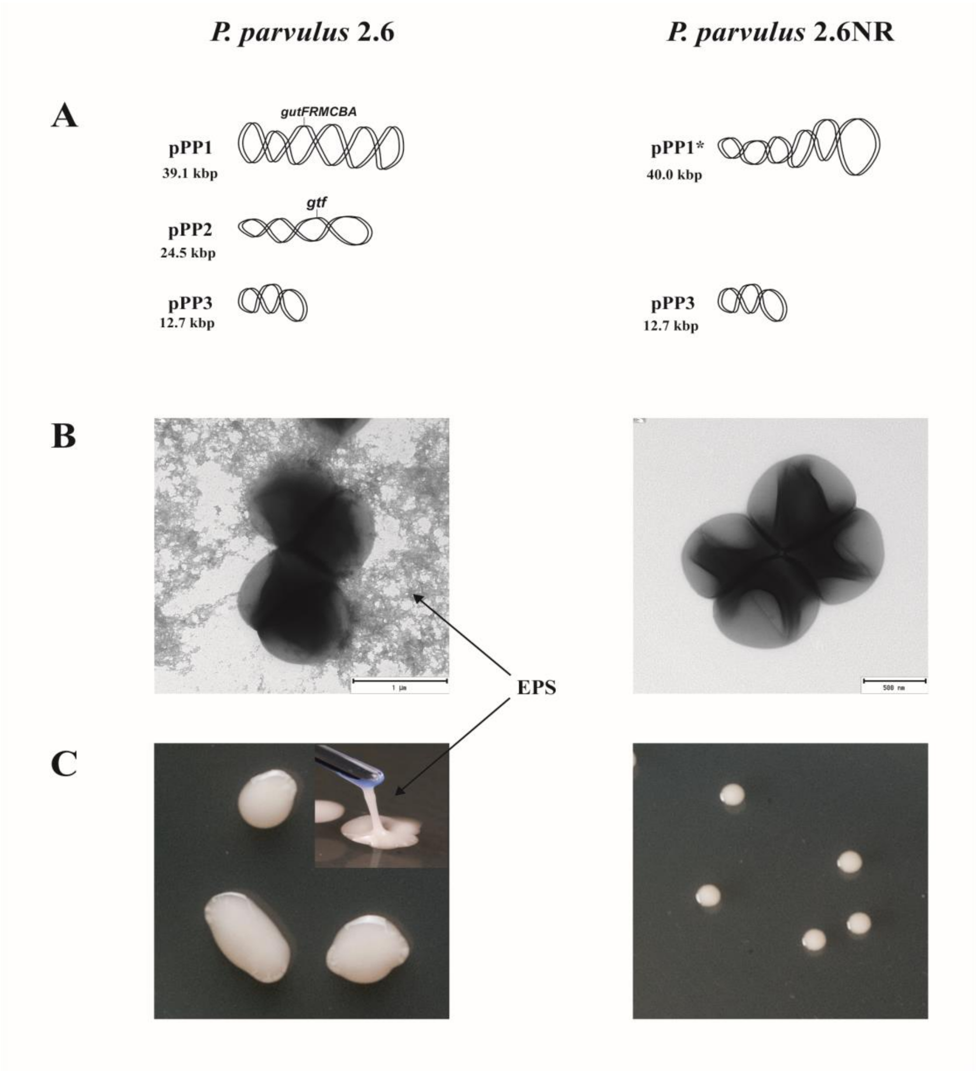


Figure 3. The *P. parvulus* 2.6 and 2.6NR, ropy and non-ropy strains. (**A**) Scheme of the plasmids harboured by the bacteria, their sizes and genes of interest are indicated. (**B**) Electron microscopic images of the bacteria. (**C**) Images of colonies of the bacteria in MRS medium, the typical ropy phenotype of the 2.6 is characteristic of EPS-producing bacteria and detected by lifting the bacteria. The presence of EPS is indicated by arrows.

The *in silico* analysis of the *P. parvulus* 2.6 genome also revealed the presence of a cluster of six genes *gutFRMCBA* (*gut* operon) involved in the transport and metabolism of sorbitol. The genetic detection of the *gut* operon by Southern blot hybridisation demonstrated that this operon is located in the pPP1 plasmid of 2.6, but not in the pPP1* plasmid of 2.6NR (Fig. 5 of Chapter 4). DNA sequencing analysis of both plasmids showed that a 9,473 bp region encompassing the *gut* operon, confirmed that is not present in pPP1* plasmid. This was an unexpected finding, since the 2.6NR strain was derived from the 2.6 strain, with the only *a priori* apparent difference being the loss of the pPP2 plasmid (Fernández et al., 1995).

The 2.6NR strain was obtained by treating the 2.6 strain with the chemicals ethidium bromide, DNA intercalating agent, and novobiocin, a bacterial gyrase inhibitor. The treatment with these compounds induce DNA mutagenesis interfering with the replication mechanisms and thus provoking curing of the plasmids (Liu et al., 2016). In the original pPP1 plasmid sequence of 2.6, a transposase and a serine recombinase are flanking the *gut* operon sequence. The region of 9 kbp could have been mobilised from the plasmid by the transposase, but if this were the case, the final product would have generated a smaller plasmid, and the pPP1* plasmid, as stated above is larger than pPP1 (40 kpb *versus* 39.1 kbp) according with its migration in agarose gels (Fig. 4 of Chapter 4). However, a serine recombinase can also act as a resolvase, catalysing the resolution of a co-integrate previously generated by recombination between two specific sites (Rice, 2015). In addition, the sequencing of pPP1* indicated the presence of a different sequence in the region of 9 kbp. Therefore, the most feasible hypothesis is that there was a recombination event between the plasmid pPP1 and another, presumably the plasmid pPP2, leading to the formation of the plasmid pPP1* and the loss of the plasmid pPP2.

2. The synthesis of the 2-substituted (1, 3)-β-glucan by *P. parvulus* 2.6

Werning et al. (2006) showed that the GTF glycosyltransferase catalyses the synthesis of the β-glucan in *P. parvulus* by usage of UDP-[^{14}C] glucose as substrate for *in vitro* synthesis of the polymer and quantifying the reaction product by measuring the radioactivity retained on Whatman GF/A filters. In Chapter 1, an *in vitro* reaction confirmed that the enzyme catalyses the synthesis of the β-glucan using UDP-glucose as substrate. The nature of the product was established by determining its monomeric composition and structure, and the quantification was performed by the specific immunological method. Thus, the overall analysis of the reaction product confirmed the specificity of the immunological method.

The metabolic pathway for the pediococcal HoPS production from glucose, fructose and maltose in 2.6 strain has been previously investigated, confirming the monomer composition and the structure of the EPS by HPLC and MNR analysis, respectively (Velasco et al., 2007). The authors described the activity of a phosphoglucose isomerase (PGI) that converts reversibly the fructose-6-P into glucose-6-P, connecting fructose to the β-glucan biosynthetic pathway (Fig. 4). Nevertheless, they found that the synthesis of the β-glucan was more effective from glucose than from fructose, the latter being a very poor substrate for the EPS synthesis. For the maltose metabolism, Velasco et al. (2007) detected two enzymatic activities: maltase and maltose phosphorylase, which generate by hydrolysis two glucose molecules or one glucose plus one β-glucose-1-P molecules, products that can enter into the β-glucan biosynthetic pathway (Fig. 4). Also in that work, it was observed that the activity of the α-phosphoglucomutase increased significantly in the presence of glucose and maltose, while in the presence of fructose the activity was low. This enzyme catalyses the reversible conversion of glucose-6-P to glucose-1-P, that communicates the sugar catabolism with the β-glucan biosynthetic pathway (Fig. 4).

The metabolic study of *P. parvulus* 2.6 performed here showed that indeed the bacterium uses this polyalcohol as an alternative carbon source for both the production of energy and the biosynthesis of the HoPS (Figs. 2 and 3 of Chapter 4). The analysis was carried out in a complex medium (MRS) and the immunological method developed in Chapter 1 was used to determine the EPS production during the bacterial growth. This method has an advantage over those previously used due to its specificity and simplicity. The polymer production took place mainly during the exponential growth phase (Fig 3 of Chapter 4), as previously described (Dueñas et al., 2003). Furthermore, the results obtained here showed that the efficiency for β-glucan production from glucose and sorbitol is similar, since equivalent amounts of the HoPS were produced per unit of biomass (Fig 3 of Chapter 4). However, the growth rate in the presence of sorbitol was drastically reduced, indicating that the bacterium does not effectively metabolize this substrate. The conversion of sorbitol-6-P to fructose-6-P needs NAD^+ as cofactor, which is reduced to NADH. A gene coding for NADH oxidase was detected in the genome of *P. parvulus* 2.6, indicating the existence of this enzyme, and its catalytic activity should improve under aerobic conditions yielding higher concentration of the NAD$^+$. Accordingly, when bacterial cultures were subjected to aeration conditions, a significant increase in bacterial growth was observed (Fig. 4 of Chapter 7). These results could indicate that the conversion to fructose-6-P by the system is not very efficient, which may promote the action of the PGI in the conversion of glucose-6-P and thus enter into the β-glucan biosynthetic pathway (Fig. 4), obtaining a similar yield in the presence of both glucose and sorbitol.

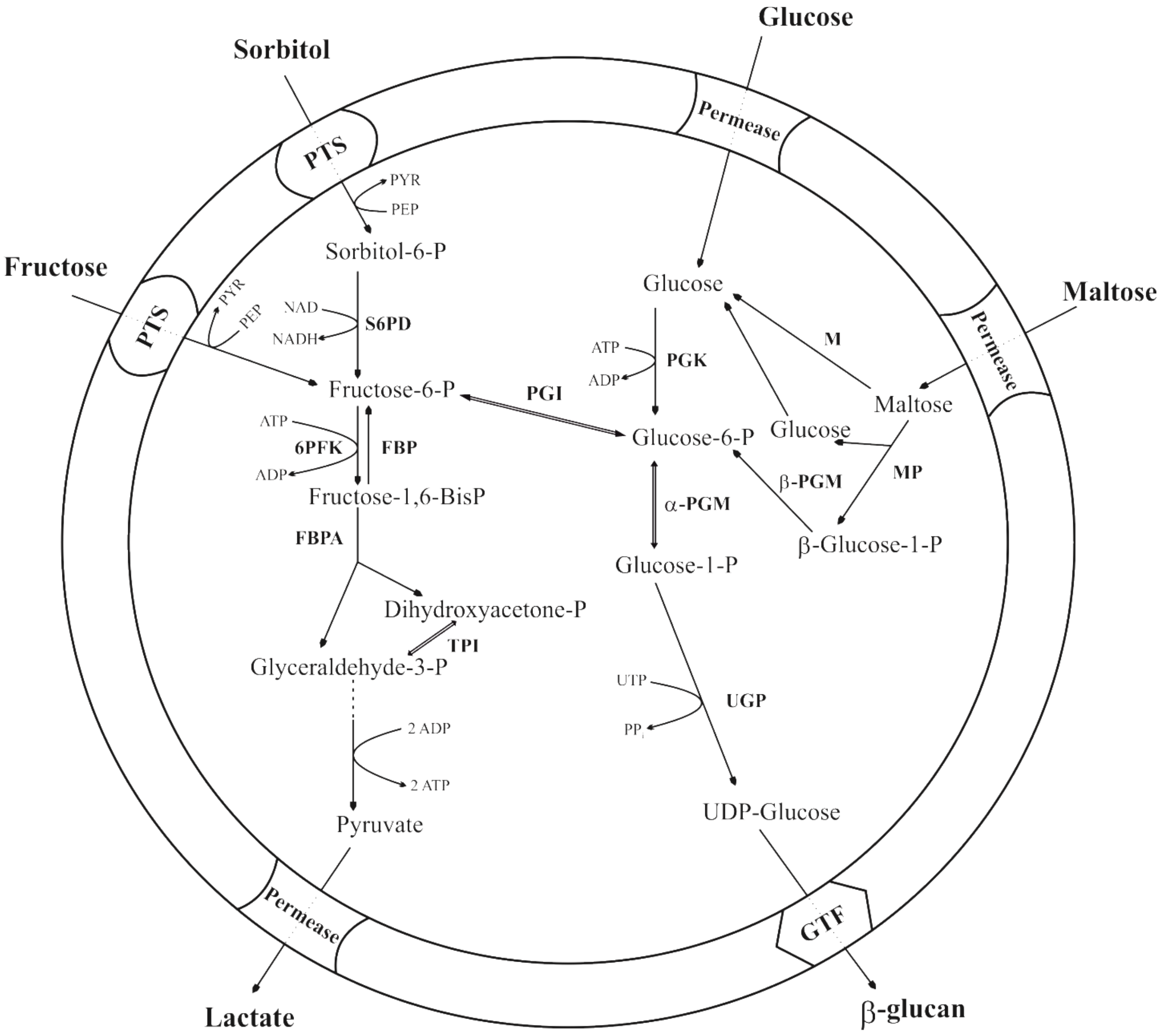


Figure 4. Schematic representation of the proposed pathway to synthesise β-glucan in *P.parvulus* 2.6. Phosphoenolpyruvate–sugar phosphotransferase system (PTS), glycosyltransferase (GTF), sorbitol-6-P dehydrogenase (S6PD), 6-phosphofructokinase (6PFK), fructose-1,6-bisphosphatase (FBP), fructose-1,6-bisphosphate aldolase (FBPA), triose-P isomerase (TPI), phosphoglucose isomerase (PGI), phosphoglucokinase (PGK), α-phosphoglucomutase (α-PGM), maltase (M), maltose phosphorylase (MP), β-phosphoglucomutase (β-PGM) and UDP-glucose pyrophosphorylase (UGP).

3. The promoter-probe vector pRCR and its utility for the analysis of regulation of expression of the *gut* operon in *P. parvulus*

In order to characterise the sorbitol utilization by *P. parvulus* 2.6, the expression of the *gut* operon was investigated in this thesis work, using the promoter-probe vector pRCR (Fig. 5 and Chapter 2). This plasmid carries the broad host range pSH71 replicon from *L. lactis*, and it contains a multicloning site upstream of the *mrfp* gene, which encodes mCherry, a monomeric variant of the red fluorescent protein of *Dicosoma* sp. (Garcia-Cayuela et al., 2012). The vector was constructed and first validated in Chapter 2 by the study of the *L. acidophilus lbaB* gene expression, cloning its P_{lbaB} promoter in the multicloning site of the vector, producing the pRCR11 plasmid (Fig. 5). The *lbaB* gene encodes the lactacin B, and its expression is regulated by the RR_1798 response regulator of a three-component system (Altermann et al., 2005; Tabasco et al., 2009), which would bind to an inverted repeat located at the promoter sequence and including the transcription initiation site, inhibiting the gene expression. The transcriptional repression is inhibited when the HK_1799 histidine kinase detects the presence of another bacterium in the extracellular medium, which triggers the phosphorylation of the repressor inactivating it. Accordingly, in this thesis an indution was observed in *L. acidophilus*[pRCR11] of the mCherry expression from P_{lbaB}, when co-cultured with *S. thermophilus* (Fig. 2 of Chapter 2).

Subsequently, other promoters have been tested in our group using the pRCR vector. The P_x promoter, which controls the expression of genes involved in the metabolism of maltosaccharides in *S. pneumoniae*, was cloned in pRCR generating the pRCR12 plasmid (Russo et al., 2015). P_x is controlled by the repressor MalR inhibiting its expression. However, outside of the pneumoccocal environment and without the presence of MalR, P_x becomes a constitutive promoter. This behaviour makes this plasmid a powerful tool for fluorescence-labelling of LAB. In this thesis work, *P. parvulus* 2.6 and 2.6NR strains have been fluorescently labelled by transfer of pRCR12 (Fig. 2 of Chapter 5). The constitutive production of mCherry from pRCR12 did not affect growth of these strains nor the β-glucan production by 2.6. The use of these recombinant bacteria in the evaluation of their probiotic properties will be discussed in section 4 of this Chapter.

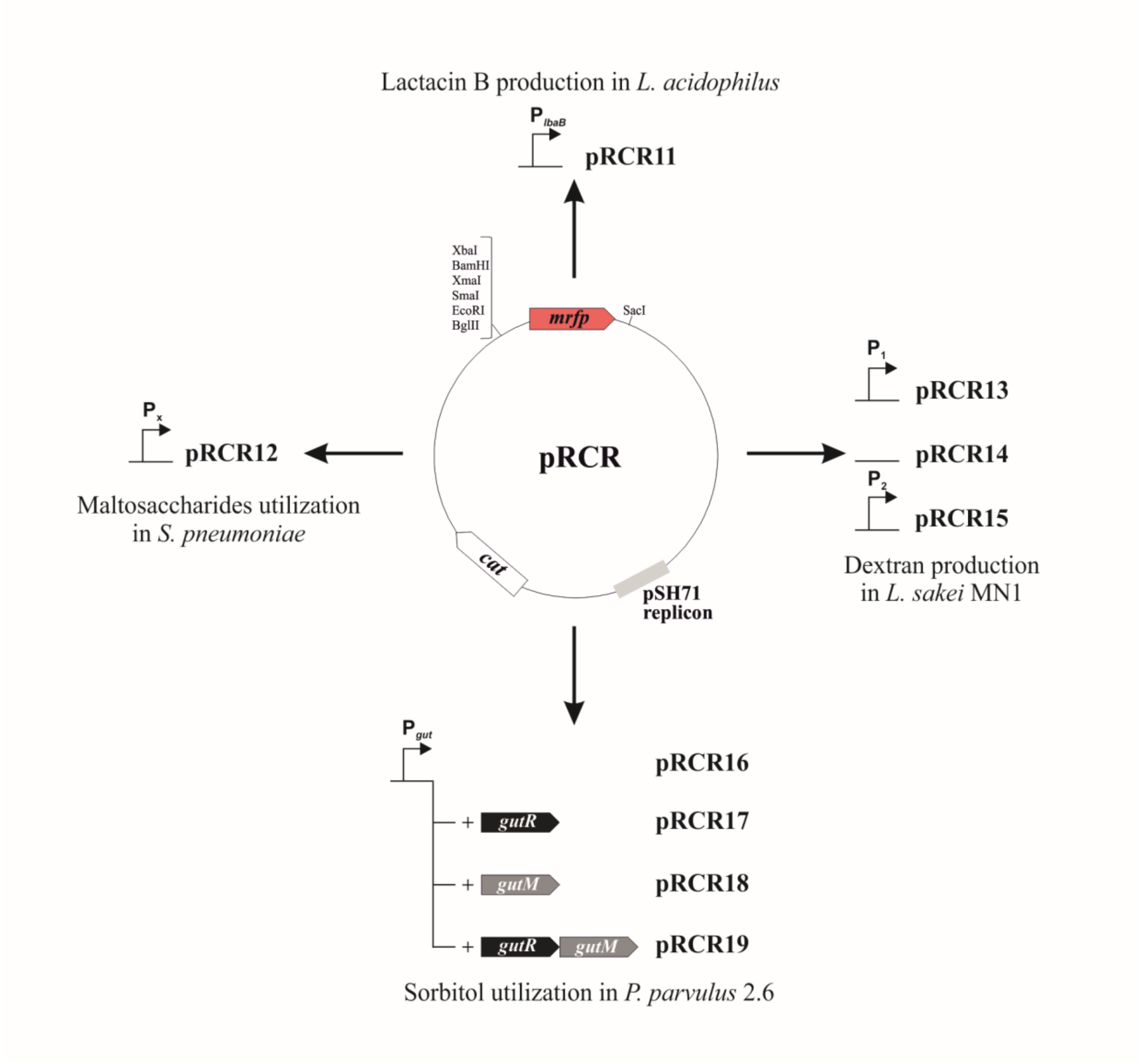
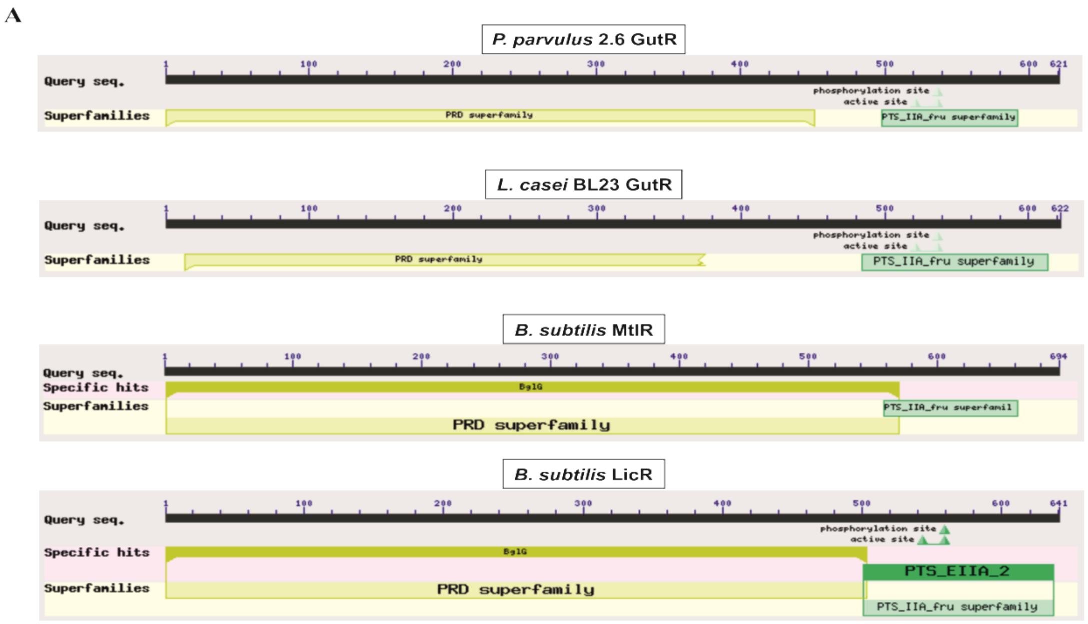


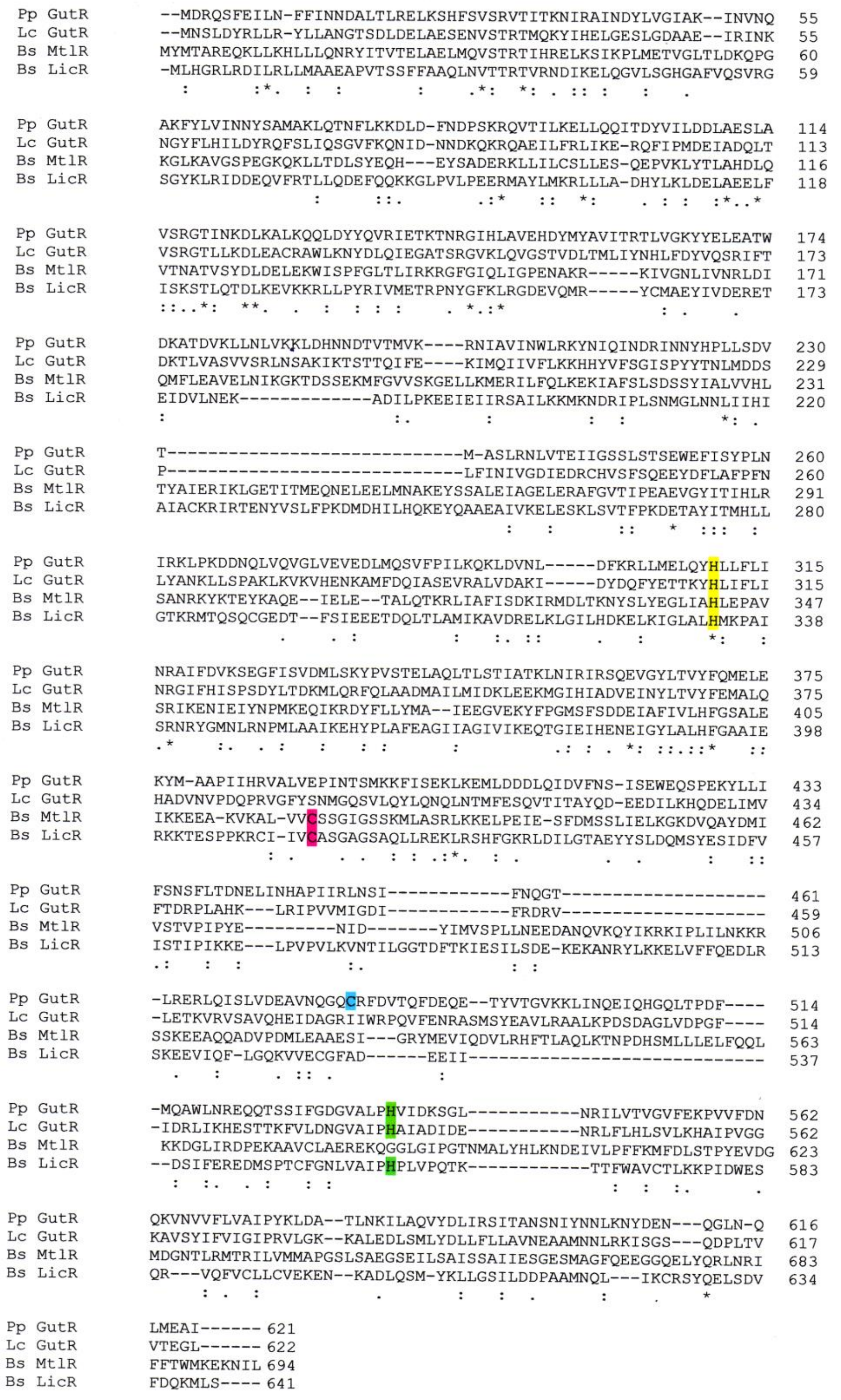
Figure 5. Scheme of the pRCR vector and its derivatives. Plasmids pRCR11-pRCR19 were generated to generate transcriptional fusions of the P_{lbaB}, P_x, P_1, P_2 and P_{gut} promoters and the *mrfp* gene. Measurements of the mCherry levels indicated the strength and activation of the promoters.

In addition, pRCR was employed to test and evaluate the putative promoter regions involved in expression of the *dsrLS* gene, which encodes a dextransucrase responsible for the production of dextran in *L. sakei* MN1 (Nácher-Vázquez et al., 2017c). Three possible promoter regions named region A, region B and region C were cloned into pRCR generating the pRCR13, pRCR14 and pRCR15 plasmids, respectively (Fig. 5). The authors detected two functional promoters, named P_1 and P_2, by the activation of mCherry expression in *L. sakei* carrying either pRCR13 or pRCR15. Also, the evaluation of the fluorescence of the recombinant bacteria showed that P_1 was stronger than P_2, and that the presence of sucrose (the substrate for dextran production) in the growth medium did not induce dextransucrase synthesis.

In this thesis, the activity of the P_{gut} promoter has been evaluated using the pRCR vector (Chapter 4). This is the first time that the functionalities of the bacterial regulatory proteins GutR and GutM have been evaluated by generating the recombinant plasmids pRCR16-pRCR19 (Fig. 5, and Fig. 1 of Chapter 4). The GutR and/or GutM coding genes were cloned upstream of the *mrfp* gene as a transcriptional fusion under the control of their natural promoter (P_{gut}). The study was carried out in two heterologous hosts that metabolise sorbitol: (i) *L. casei* BL23 whose *gut* operon has been well characterized, but it has low homology with the operon of *P. parvulus* 2.6 (Chapter 4) and, (ii) two *L. plantarum* strains: 90 (Chapter 4) and WFS1 (Chapter 7) strains, whose operons have not been characterized but have a 98% homology with that of *Pediococcus*. The results revealed a *trans*-complementation of the *L. plantarum* regulatory proteins on expression driven from the *P. parvulus* P_{gut} promoter, in cultures grown in the presence of sorbitol. Moreover, the results indicate that in the presence of glucose a catabolic repression controlling expression from P_{gut} takes place. Supporting this hypothesis, a *cre* sequence is located downstream and next to P_{gut} (see Fig. 7). Furthermore, in *L. casei* the presence of sorbitol in the growth medium and the pediococcal *gutR* in the genome was required for P_{gut} functionality, and therefore that GutR of 2.6 acts as a transcriptional activator.

Blast analysis (Fig. 6A) and clustal multialignment (Fig. 6B) revealed that *P. parvulus* 2.6 GutR belongs to the family of transcriptional activators that include GutR of *L. casei* BL23 as well as MtlR and LicR of *B. subtilis,* which control the PTS transport and metabolism of oligomeric β-glucosides and of mannitol, respectively. The regulators contain the PDR and PTS_EII domains, which contain phosphorylateable His or Cys residues (Tobisch et al., 1999; Alcántara et al., 2008; Joyet et al., 2013; Deutscher et al., 2014). Moreover, after phosphorylation at one of these residues located at the PDR domain the regulators become active by dimerization or inactive by phosphorylation at the PTS_EII domain (Joyet et al., 2013). In *L. casei*, it seems that when P-His-HPr phosphorylates His^{310} of GutR, triggers its activation (Alcántara et al., 2008). The multialignment depicted in Figure 6B shows conservation of this residue at positions 310, 342 and 333 in GutR of 2.6, MtlR and LicR, respectively, supporting the notion that the His^{310} residue is the phosphorylatable residue involved in activation, as in other regulators (Deutscher et al., 2014). In *L. casei* GutR, phosphorylation of its His^{537} by EIIBC triggers its inactivation (Alcántara et al., 2008). Moreover in LicR, mutagenic analysis indicates that the His^{559} is the key residue involved in inactivation (Tobisch et al., 1999). The equivalent residue His^{537} is present in GutR of *P. parvulus* and it is missing in MtlR (Fig. 6B). Thus, MtlR is subject to a different mechanism of modification. It is negatively regulated by EIIA, which phosphorylates its Cys^{419}(Bouraoui et al., 2013). The equivalent residue is present in LicR (Cys^{413}) (Fig. 6B) and it can have also a role in this activator. In addition, it has been reported that MtlR needs to interact with EIIB for its activation. Also, EIIB has to sequester MtlR to the membrane for its activation, since interaction with soluble EIIB in the cytoplasm does not trigger MtlR activation (Bouraoui et al., 2013). Furthermore, this interaction occurs only with the unphosphorylated form of EIIB. If this is phosphorylated the interaction does not take place (Joyet et al., 2013). GutR of 2.6, but not of *L. casei* BL23, has a potential phosphorylable Cys^{480} like the MtlR regulator. Nevertheless, this Cys^{480} is out of the EIIA domain of the protein, and does not align with the Cys residues of the *B. subtilis* regulators (Fig. 6B). Therefore, inactivation of 2.6 GutR could be regulated by EIIBC, in a way similar to that postulated for *L. casei*.





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Pp GutR    --MDRQSFEILN-FFINNDALTLRELKSHFSVSRVTITKNIRAINDYLVGIAK--INVNQ   55
Lc GutR    --MNSLDYRLLR-YLLANGTSDLDELAESENVSTRTMQKYIHELGESLGDAAE--IRINK   55
Bs MtlR    MYMTAREQKLLKHLLLQNRYITVTELAELMQVSTRTIHRELKSIKPLMETVGLTLDKQPG   60
Bs LicR    -MLHGRLRDILRLLMAAEAPVTSSFFAAQLNVTTRTVRNDIKELQGVLSGHGAFVQSVRG   59
             :      :*.  :  :       :    .*:  *: . :: :   :   .

Pp GutR    AKFYLVINNYSAMAKLQTNFLKKDLD-FNDPSKRQVTILKELLQQITDYVILDDLAESLA  114
Lc GutR    NGYFLHILDYRQFSLIQSGVFKQNID-NNDKQKRQAEILFRLIKE-RQFIPMDEIADQLT  113
Bs MtlR    KGLKAVGSPEGKQKLLTDLSYEQH---EYSADERKLLILCSLLES-QEPVKLYTLAHDLQ  116
Bs LicR    SGYKLRIDDEQVFRTLLQDEFQQKKGLPVLPEERMAYLMKRLLLA-DHYLKLDELAEELF  118
                          :     ::.       .:*   ::  *:    . : :  :*..*

Pp GutR    VSRGTINKDLKALKQQLDYYQVRIETKTNRGIHLAVEHDYMYAVITRTLVGKYYELEATW  174
Lc GutR    VSRGTLLKDLEACRAWLKNYDLQIEGATSRGVKLQVGSTVDLTMLIYNHLFDYVQSRIFT  173
Bs MtlR    VTNATVSYDLDELEKWISPFGLTLIRKRGFGIQLIGPENAKR-----KIVGNLIVNRLDI  171
Bs LicR    ISKSTLQTDLKEVKKRLLPYRIVMETRPNYGFKLRGDEVQMR-----YCMAEYIVDERET  173
           ::..*:  **.  .  :  : : :    . *.:*               : .    .

Pp GutR    DKATDVKLLNLVKKLDHNNDTVTMVK----RNIAVINWLRKYNIQINDRINNYHPLLSDV  230
Lc GutR    DKTLVASVVSRLNSAKIKTSTTQIFE----KIMQIIVFLKKHHYVFSGISPYYTNLMDDS  229
Bs MtlR    QMFLEAVELNIKGKTDSSEKMFGVVSKGELLKMERILFQLKEKIAFSLSDSSYIALVVHL  231
Bs LicR    EIDVLNEK-------------ADILPKEEIEIIRSAILKKMKNDRIPLSNMGLNNLIIHI  220
           :                      :.       :         :  :         *: .

Pp GutR    T-----------------------------M-ASLRNLVTEIIGSSLSTSEWEFISYPLN  260
Lc GutR    P-----------------------------LFINIVGDIEDRCHVSFSQEEYDFLAFPFN  260
Bs MtlR    TYAIERIKLGETITMEQNELEELMNAKEYSSALEIAGELERAFGVTIPEAEVGYITIHLR  291
Bs LicR    AIACKRIRTENYVSLFPKDMDHILHQKEYQAAEAIVKELESKLSVTFPKDETAYITMHLL  280
                                             :   :      ::   *  :::  :

Pp GutR    IRKLPKDDNQLVQVGLVEVEDLMQSVFPILKQKLDVNL-----DFKRLLMELQYHLLFLI  315
Lc GutR    LYANKLLSPAKLKVKVHENKAMFDQIASEVRALVDAKI-----DYDQFYETTKYHLIFLI  315
Bs MtlR    SANRKYKTEYKAQE--IELE--TALQTKRLIAFISDKIRMDLTKNYSLYEGLIAHLEPAV  347
Bs LicR    GTKRMTQSQCGEDT--FSIEEETDQLTLAMIKAVDRELKLGILHDKELKIGLALHMKPAI  338
                       .    . :         :   :. ::     .   :      *:   :

Pp GutR    NRAIFDVKSEGFISVDMLSKYPVSTELAQLTLSTIATKLNIRIRSQEVGYLTVYFQMELE  375
Lc GutR    NRGIFHISPSDYLTDKMLQRFQLAADMAILMIDKLEEKMGIHIADVEINYLTVYFEMALQ  375
Bs MtlR    SRIKENIEIYNPMKEQIKRDYFLLYMA--IEEGVEKYFPGMSFSDDEIAFIVLHFGSALE  405
Bs LicR    SRNRYGMNLRNPMLAAIKEHYPLAFEAGIIAGIVIKEQTGIEIHENEIGYLALHFGAAIE  398
           .*    :.  . :   :   : :      :         .: : . *: ::.::*   ::

Pp GutR    KYM-AAPIIHRVALVEPINTSMKKFISEKLKEMLDDDLQIDVFNS-ISEWEQSPEKYLLI  433
Lc GutR    HADVNVPDQPRVGFYSNMGQSVLQYLQNQLNTMFESQVTITAYQD-EEDILKHQDELIMV  434
Bs MtlR    IKKEEA-KVKAL-VVCSSGIGSSKMLASRLKKELPEIE-SFDMSSLIELKGKDVQAYDMI  462
Bs LicR    RKKTESPPKRCI-IVCASGAGSAQLLREKLRSHFGKRLDILGTAEYYSLDQMSYESIDFV  457
                      : .    . .  : : .:*.  : .        .  .      :   ::

Pp GutR    FSNSFLTDNELINHAPIIRLNSI------------FNQGT--------------------  461
Lc GutR    FTDRPLAHK---LRIPVVMIGDI------------FRDRV--------------------  459
Bs MtlR    VSTVPIPYE---------NID-------YIMVSPLLNEEDANQVKQYIKRKIPLILNKKR  506
Bs LicR    ISTIPIKKE---LPVPVLKVNTILGGTDFTKIESILSDE-KEKANRYLKKELVFFQEDLR  513
           .:   :  :          :.              : :

Pp GutR    -LRERLQISLVDEAVNQGQCRFDVTQFDEQE--TYVTGVKKLINQEIQHGQLTPDF----  514
Lc GutR    -LETKVRVSAVQHEIDAGRIIWRPQVFENRASMSYEAVLRAALKPDSDAGLVDPGF----  514
Bs MtlR    SSKEEAQQADVPDMLEAAESI---GRYMEVIQDVLRHFTLAQLKTNPDHSMLLLELFQQL  563
Bs LicR    SKEEVIQF-LGQKVVECGFAD------EEII-----------------------------  537
             .   :     . :: .          :

Pp GutR    -MQAWLNREQQTSSIFGDGVALPHVIDKSGL-----------NRILVTVGVFEKPVVFDN  562
Lc GutR    -IDRLIKHESTTKFVLDNGVAIPHAIADIDE-----------NRLFLHLSVLKHAIPVGG  562
Bs MtlR     KKDGLIRDPEKAAVCLAEREKQGGLGIPGTNMALYHLKNDEIVLPFFKMFDLSTPYEVDG  623
Bs LicR    --DSIFEREDMSPTCFGNLVAIPHPLVPQTK------------TTFWAVCTLKKPIDWES  583
             :  :.  . :   : :                           :  :  :.      .

Pp GutR    QKVNVVFLVAIPYKLDA--TLNKILAQVYDLIRSITANSNIYNNLKNYDEN---QGLN-Q  616
Lc GutR    KAVSYIFVIGIPRVLGK--KALEDLSMLYDLLFLLAVNEAAMNNLRKISGS---QDPLTV  617
Bs MtlR    MDGNTLRMTRILVMMAPGSLSAEGSEILSAISSAIIESGESMAGFQEEGGQELYQRLNRI  683
Bs LicR    QR---VQFVCLLCVEKEN--KADLQSM-YKLLGSILDDPAAMNQL---IKCRSYQELSDV  634
                : .  :           .       :   :  .      :    .    *

Pp GutR    LMEAI------ 621
Lc GutR    VTEGL------ 622
Bs MtlR    FFTWMKEKNIL 694
Bs LicR    FDQKMLS---- 641
```

Figure 6. Regulators involved in genetic activation of the PTS systems. (A) Prediction of the PDR and PTS phosphorylation domains of the proteins by BLAST analysis. (B) CLUSTAL 2.1 multiple alignment of the protein amino acid sequences. Background colours of the amino acids: His residues predicted to be phosphorylated by HprK (yellow); His (green) or Cys (violet) residues predicted to be phosphorylated by phosphotransfer from HPr; Cys (blue) residue present in *P. parvulus* GutR and not conserved in the other proteins. Proteins: *P. parvulus* 2.6 GutR (*P. parvulus* GutR), *L. casei* BL23 GutR (*Lc* GutR), *B. subtilis* MtlR (Bs MtlR) or (Bs LicR) regulators. Symbols: (*) indicates positions which have a single, fully conserved residue; (:) indicates conservation between groups of strongly similar properties, equivalent to scoring > 0.5 in the Gonnet PAM 250 matrix; (.) indicates conservation between groups of weakly similar properties, equivalent to scoring =< 0.5 and > 0 in the Gonnet PAM 250 matrix.

Furthermore, it has been postulated that in *L. casei*, GutM should favour the activation of transcription of the *gut* operon, but the mechanism of action is unknown (Alcántara et al., 2008). The results obtained in this thesis work revealed that the overexpression of GutM of 2.6 in *L. plantarum* provoked the highest induction of expression from P_{gut} (Fig. 8 and Table 4 of Chapter 4). Thus, this result suggests: (i) that GutM has a role in the regulation of the *gut* operon and (ii) that a protein-protein interaction between the *P. parvulus* GutM and the *L. plantarum* GutR could potentiate the activation of the P_{gut} promoter. Nonetheless, in *L. casei* and *L. plantarum* a decrease of expression from P_{gut} was observed when GutM was overexpressed in combination with GutR. Moreover, *in silico* analysis of the overlapping regions of *gutR* and *gutM* suggested a possible phenomenon of post-transcriptional regulation by means of a -1 frameshift translation, which may lead to the synthesis of a fused protein GutR-M (Fig. 9 of Chapter 4). Presumably, this putative fused protein would occur at a low rate and only in the presence of both genes in a high copy plasmid in the heterologous systems. The existence of GutR-M and if the fused protein has a role in lactobacilli, could explain the antagonistic effect of overexpression of the pediococcal *gutM* gene alone or together with the *gutR* gene (Fig. 8 and Tables 3 and 4 of Chapter 4). Furthermore, the analysis of the amino acid sequence of GutM predicted a transmembrane region in the N-terminal, thus presumably GutM is a protein anchored to the membrane. Therefore, GutR fused or bound to GutM would be recruited to the membrane.

Finally, based on the overall results presented here and the current knowledge, I propose the following model of regulation of expression of the *gut* operon in *P. parvulus* 2.6 (Fig. 7). In the presence of glucose, the ratio PEP/PYR would be low, so the autophosphorylation of EI from PEP should be reduced, therefore the phosphorylation cascade to the EIIgut domains would be inhibited. In addition, the increase of the intermediate fructose-1,6-bisphosphate (FBP) metabolite would promote the generation of the P-Ser-HPr form by the kinase activity of HprK/P. P-Ser-HPr will interact with CcpA, and this complex will repress gene expression from P_{gut} by binding to the *cre* site. Presumably, GutR will be recruited to the membrane by GutM, favouring its inactivation by P-EIIBC (Fig. 6A). In the presence of sorbitol and without glucose, the level of FBP will decrease and the level of inorganic phosphate will increase, promoting the phospholyration activity of HprK/P that produces dephosphorylation of P-Ser-HPr. In addition, the ratio PEP/PYR will increase and the autophosphorylation of EI would be activated, triggering the phosphorylation cascade from P-His-HPr to the EII domains. In this condition, EIIBC would transfer its phosphate to the sorbitol instead of transferring to GutR. Thus, GutR would be activated by P-His-HPr, promoting its dimerization, its further binding to its operator located upstream of P_{gut} and provoking induction of the *gut* operon expression (Fig. 6B).

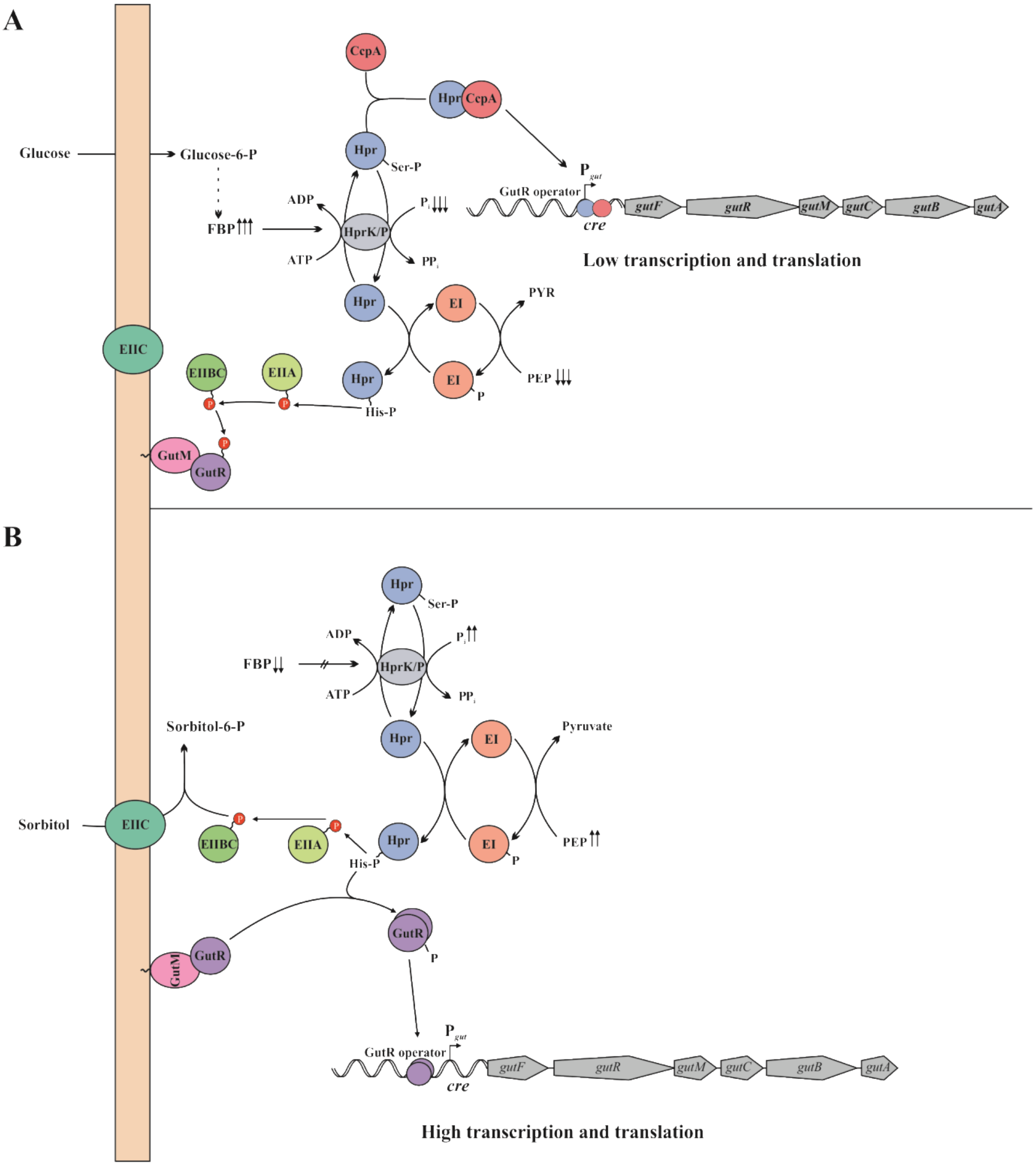

Figure 7. Model for the regulation of the *gut* operon of *P. parvulus*, in the absence (A) or in the presence of sorbitol (B).

4. Probiotic properties of *P. parvulus* 2.6

The characterisation of the probiotic properties of *P. parvulus* 2.6 has been pursued for years. In this work, analysis of the *P. parvulus* genome, determined in Chapter 2, revealed that it does not contain any genetic determinant for the production of biogenic amines, undesirable compounds in fermented foods and beverages (Mohedano et al., 2015b). Moreover, production of putrescine, histamine and tyramine by the 2.6 strain has been tested using thin-layer chromatography and the compounds were not detected (unpublished results of Mª Goretti Llamas).

Transmissible antibiotic resistance is a property to be strictly avoided in probiotics, due to the risk of horizontal transfer of the genetic determinants to other bacteria. Thus, the 2.6 genome was analysed and no genetic determinants for resistance to antibiotics were detected. In addition, the antibiotic resistance profile of *P. parvulus* 2.6 (Supplementary Table S1 of Chapter 5) was performed according to the recommendations of EFSA, and the following antibiotics were tested: ampicillin, chloramphenicol, clindamycin, erythromycin, gentamycin, kanamycin, streptomycin, tetracycline, and vancomycin. The minimal inhibitory concentration of all the antibiotic tested were under the cut-off values determined by EFSA (EFSA, 2012), thus 2.6 is sensitive to all of them. In addition, the *P. parvulus* species was catalogued with the QPS status and included in the official list of the EFSA in June 2016 (Ricci et al., 2017), supporting the safety of this bacterium for its use in animal and human health.

An essential requirement of a probiotic is to remain viable until it reaches the environment, mainly the colon, where it exerts its beneficial effect. This entails facing the acid and enzymatic stress conditions of the upper digestive tract, both at an oral level resisting the action of lysozyme, and at a gastric level supporting acidity that can reach pH 2.0 or lower, as well as at an intestinal level where probiotics must resist the action of bile salts and pancreatic juices. The EPS form a polysaccharidic matrix that envelops the producing bacteria and can create a protective environment against these stresses. There are some works that report a beneficial influence of the EPS on the resistance of producing bacteria to gastrointestinal stress (Alp et al., 2010; Dertli et al., 2015), and other studies do not report a direct influence of an EPS (Nikolic et al., 2012).

In this thesis, the resistance conferred by the β-glucan of *P. parvulus* was evaluated. *L. plantarum* WCFS1, included into the cereal-based foods fortified with the pediococcal β-glucan, was subjected to an *in vitro* simulated GIT stress (Fig. 3 from Chapter 6). The bacterial EPS present in the oat matrix conferred to WCFS1 resistance to bile salts and pancreatin of the intestinal stress. Previously, Stack et al. (2010) expressed in multicopy the *gtf* gene of *P. parvulus* 2.6 in *Lactobacillus paracasei* NFBC 338, producing heterologously the β-glucan. The presence of the EPS conferred to *L. paracasei* 15-fold and 5.5-fold increase in survival under simulate gastric juice stress and bile stress, respectively. The same effect of the EPS was observed when, in this case, the survival of *P. parvulus* 2.6 and 2.6NR strains through the GIT was evaluated using an *in vivo* mouse model (Lindström et al., 2012). By contrast, Fernández de Palencia et al. (2009) did not observe any influence of the β-glucan when *P. parvulus* 2.6 and 2.6NR strains were subjected *in vitro* to simulated GIT stress.

In Chapter 7 of this work the 2.6 and 2.6NR strains were subjected to other stresses relavant to food manufacturing. Although the bacteria can tolerate oxidative or salinity stresses, the influence of the β-glucan was only detected for the ethanol stress (Fig. 2 and Table 1 of Chapter 7). In the 2.6 strain, β-glucan protected the bacterium improving its survival in the presence of ethanol and, in addition, the production of the EPS increased.

Once probiotics viably reach their target environment they need time to further adapt. For this reason, the ability to adhere to the intestinal epithelium promotes the persistence of these probiotics in the GIT, allowing them to exert their action. Thus, the adhesion capacity of probiotics is one of the most important criteria for their selection. In this thesis the adhesive capability of the recombinant *P. parvulus* 2.6[pRCR12] (2.6p) and the 2.6NR[pRCR12] (2.6NRp) strains was assayed *in vitro* by testing bacterial binding to Caco-2 cells. The 2.6p strain showed a higher adhesion than 2.6NRp (Fig. 3 in Chapter 5), demonstrating the positive contribution of the bacterial β-glucan described previously for *P. parvulus* 2.6 (Fernández de Palencia et al., 2009). Similar results were reported for the β-glucan-producing strain *P. parvulus* CUPV22, whose adhesion to Caco-2 cells was decreased when the EPS was mechanically removed from the cells surface (Garai-Ibabe et al., 2010). In addition, for other bacteria belonging to other genera such as *P. freudenreichii* and *O. oeni*, it has been suggested that the presence of this bacterial β-glucan influences their adhesion capacity (Nordmark et al., 2005; Dols-Lafargue et al., 2008).

Furthermore, in Chapter 5 of this work the quantity of β-glucan attached to the 2.6 strain (0.25 mg mL^{-1}), during the binding assays to Caco-2 cells, was determined by the immunological method. The addition of this amount of purified β-glucan to bacterial solution of 2.6NRp leaded to a 5.5-fold increase in its adhesion capability to enterocytes (Fig. 3 in Chapter 5). The increase of the amount of β-glucan added did not lead to an increase in bacterial adhesion (results not shown). Russo et al. (2012) reported the same adhesion increase for *L. plantarum* WCFS1, but upon addition of 5 mg mL^{-1} of the purified β-glucan.

The overall results showed the positive influence of the pediococcal β-glucan in bacterial adhesion to enterocytes. But, in the literature the influence of various types of EPS on this property is controversial. The HePS, ESP-SJ, produced by *L paracasei* subsp. *paracasei* BGSJ2-8 conferred better adhesion capacity to the bacterium in a comparative study with a non-producing mutant (Živković et al., 2016). Other studies have reported that EPS-producing bacteria have a high adhesion capacity, but they have not proven the direct influence of the EPS on this property (Alp et al., 2010; García-Ruiz et al., 2014). On the contrary, other studies have reported the negative influence of the EPS on adhesion of the producing bacteria to epithelial cells. A HePS non-producing mutant of *L. johnsonii* FI9785 showed an increased adhesion capability to human HT29 cells (Horn et al., 2013) and to chicken gut explants (Dertli et al., 2015). The same effect was reported for the dextran-producing *Lactobacillus sakei* MN1. This bacterium produces its EPS only in the presence of sucrose, and when it was grown in glucose (non-EPS production) showed a greater capacity of adhesion to Caco-2 cells (Nácher-Vázquez et al., 2017b). Therefore, the removal of the EPS might enhance bacterial attachment, exposing adhesins and/or other cell surface factors that favour the process of bacterial adherence. In addition, other EPS produced by *L. rhamnosus* (Lebeer et al., 2009; Polak-Berecka et al., 2014), *L. salivarius* (Mercan et al., 2015), *Lactobacillus paraplantarum* (Nikolic et al., 2012), *L. mesenteroides* (Nácher-Vázquez et al., 2017b; Zarour et al., 2017) or bifidobacteria (Ruas-Madiedo et al., 2006) have been reported to reduce bacterial adhesion capability to intestinal epithelial cells. Furthermore, there are cases where the presence of the EPS did not affect the adhesion capacity of the producing strains (Ruas-Madiedo et al., 2006; Zarour et al., 2017).

To further investigate the influence of the β-glucan, the ability of *P. parvulus* to colonise *in vivo* the zebrafish GIT has been investigated (Fig. 4 of Chapter 5). The results showed that 2.6p colonised better the zebrafish epithelium than that of its isogenic strain 2.6NR. These *in vivo* results validated those obtained in the *in vitro* models, and confirm the positive effect of the β-glucan on the bacterial adhesion. Also, the zebrafish model was used previously to confirm that the production of dextran by *L. sakei* MN1 reduces the adhesion capacity of the bacteria, and therefore restrains the colonisation of zebrafish GIT (Nácher-Vázquez et al., 2017b). Furthermore, the fluorescent-labelling of *P. parvulus* strains allowed monitoring *in vivo* of the bacterial colonisation by the detection of the mCherry signal inside the larvae using fluorescence microscopy (Fig, 5 of Chapter 5). In addition, the plasmid pRCR12 has been already validated as a useful tool for labelling and colonization studies of *L. plantarum*, *L. fermentum* and *L. sakei* (Russo et al., 2015; Nácher-Vázquez et al., 2017b). In addition, the use of other fluorophores such as the green fluorescent protein GFP has been validated for the study of zebrafish colonization by *L. casei* (Rieu et al., 2014). However, the use of fluorescent markers is not essential for *in vivo* colonization studies in zebrafish, since we have recently evaluated non-labelled strains of *L. mesenteroides* (Zarour et al., 2018).

One of the most important uses given to probiotics is their ability to fight or prevent pathogen infection. Thus, probiotics are of importance with regard to the aim of stopping the indiscriminate use of antibiotics in the livestock and aquaculture industries for weight gain and prevention of epidemics, which leads to the emergence of resistant pathogen strains that can be transmitted both to other animals and to humans (FAO, 2016). In the long term, this can cause economic losses and health problems. Many enteric pathogens such as *E. coli* or *L. monocytogenes* need to interact with the host's intestinal epithelium to establish their infection. Thereby, the adhesion of probiotics to these epithelial cells can displace or block the binding of pathogens and prevent their infection (Westermann et al., 2016). With the aim of studying pathogen inhibition by probiotic bacteria, zebrafish could be a suitable model (Rendueles et al., 2012).

For the first time, and in this work, the ability of *P. parvulus* 2.6 to fight the infection produced by pathogens was tested (Fig. 6 of Chapter 5). An *in vivo* zebrafish model was used to analyse the activity of the bacterium against infection induced by the specific fish pathogen *Vibrio anguillarum*. Thus, larvae were first colonised by bacterial solutions of *P. parvulus* strains and then were infected by exposure to bacterial solutions of *V. anguillarum*. Larvae pre-treated with 2.6p bacterial solutions responded better to *Vibrio* infection than those pre-treated with 2.6NRp. Therefore, the higher colonization capacity of the β-glucan-producing 2.6 strain was related to its protective effect. The results obtained suggest that the mechanism of action of this bacterium is based on the steric impediment to adhere to the intestinal epithelium of the zebrafish, blocking the pathogen binding sites. This protective effect against *V. anguillarum* infection in zebrafish has been reported for *L. sakei* MN1, and also under conditions that improve adhesion of the LAB to the epithelium a higher anti-infective effect was detected (Nácher-Vázquez et al., 2017b).

5. The 2-substituted (1, 3)-β-glucan as immunomodulator

In this thesis work, the immunomodulation properties of the β-glucan of *P. parvulus* has been analysed in an *in vivo* zebrafish model (Figs. 7 and 8 of Chapter 5). The results obtained revealed that the exposure of the gnotobiotic larvae to the β-glucan purified by molecular exclusion chromatography (Notararigo et al., 2013) did not affect the level of expression genes encoding TLR2, TLR22 and TLR4 receptors of immune zebrafish. TLRs have been described to be involved in β-glucan recognition (Leulier and Lemaitre, 2008). In addition, Dectin-1 is the best characterised β-glucan receptor (Brown, 2005) and in previous works, the ability of the β-glucan to bind to Dectin-1 and trigger its signalling pathway was tested *in vitro*, but there was no recognition by this receptor (Notararigo, 2015). The fact that the expression of TLRs is not induced in the presence of the β-glucan, and that dectin-1 alone does not recognize the β-glucan, does not mean that they cannot be involved in its recognition. Maybe, the receptor involved in the recognition of *P. parvulus* β-glucan consists in a heterodimer formed by units of some of these receptors. Nevertheless, further experiments are needed to determine if this hypothesis is true.

Nevertheless, the analysis of gene expression in gnotobiotic zebrafish larvae exposed to β-glucan showed a repression of gene expression of two pro-inflammatory cytokines, TNF-α and IL8 (Fig. 7 of Chapter 5). In addition, the repressed expression of the protein adaptor MyD88 was co-expressed, which mediates activation of pro-inflammatory cytokines via NF-kB. These results correlated with those previously obtained *in vitro* from immunomodulation assays conducted on monocyte-derived macrophages with 2.6 and 2.6NR strains (Fernández de Palencia et al., 2009). The authors showed that the 2.6 strain induced less production of TNF-α and IL8 and higher production of IL10 than 2.6NR strain. Thus, the presence of the β-glucan favoured a lower pro-inflammatory response. The same influence of this HoPS counteracting the pro-inflammatory effect of *P. parvulus* was described when testing in a mouse model (Lindström et al., 2012).

However, when the researchers treated the mice with the purified β-glucan they observed no response (Lindström et al., 2012). These contradictory results prompted Notararigo et al. (2014) to use the purified β-glucan as well as the pro-inflammatory *E. coli* lipopolysaccharide (LPS) to stimulate production of cytokines by human macrophages *in vitro*. The authors observed that pediococcal EPS did not induce the JNK and p38 signalling pro-inflammatory pathways in M1 macrophages. In addition, the ratio TNFα/IL10, that indicates an induction of pro-inflammatory response, was higher in the presence of LPS than of β-glucan. Also, the production of anti-inflammatory IL10 was induced to a greater extent by the pediococcal HoPS, indicating the anti-inflammatory potential of this polymer (Notararigo et al., 2014).

Thus, to try to confirm this effect *in vivo*, in this thesis work, the purified polymer of the 2.6 strain was tested in an induced inflammation zebrafish model, using the *Tg*(*mpx:GFP*) zebrafish line, whose neutrophils express constitutively the green fluorescent protein. In this assessment, an anti-inflammatory effect of the β-glucan was observed, reducing the recruitment and proliferation of the neutrophils of the treated larvae (Fig. 8 of Chapter 5). Neutrophil are recruited to the inflammation zone by the action of the chemokine like IL8 (de Oliveira et al., 2013). Also, the usage of the pediococcal β-glucan in an *ex vivo* model of biopsy colonic tissue from patients suffering from Crohn's disease, resulted in the inhibition of IL8 secretion (Notararigo, 2015). All this evidence indicates that the β-glucan of *P. parvulus* 2.6 exerts its anti-inflammatory response via the IL8 pathway.

6. The usage of *P. parvulus* 2.6 for the production of functional food

The results obtained in this thesis work support that both *P. parvulus* 2.6 and its β-glucan could provide health benefits. Thus, this bacterium could be used for the production of functional foods. The fermented foods are important vehicles for the delivery of probiotic bacteria, and the cereal-based fermented products have emerged as significant ones (Russo et al., 2016). Historically, the most important fermented foods are the dairy products, but due to the raising lactose intolerance and allergies to milk proteins the cereal-based foods entered into the market with great acceptance (Gawkowski and Chikindas, 2012; Vijaya Kumar et al., 2015).

A previous work already showed that this bacterium could ferment oat-based matrices, and that the administration of this food in human resulted in an increase of the bifidobacteria content and in a reduction in blood cholesterol level (Mårtensson et al., 2005). Moreover, the results presented here (Chapter 6) have shown how *P. parvulus***,** besides fermenting oat matrix, is capable of fermenting rice matrix. Also, in this work, it has been possible to evaluate *in situ* the production of pediococcal β-glucan in food matrices due to the use of the immunological method developed in Chapter 1. Neither the carbohydrates nor even the oat's β-glucans interfered with the detection and quantification of the β-glucan synthesised by *P. parvulus*. Although the bacterium is capable of fermenting both matrices, it ferments the oat matrix more effectively and is capable of producing greater amounts of EPS, resulting in a higher viscosity confer to the matrix (Fig. 1 and Tables 1 and 2 of Chapter 6).

Cereal products such as oats have important benefits for the health, since oat β-glucans have been reported to exert beneficial effects on insulin resistance, dyslipidaemia, hypertension and obesity (Daou and Zhang, 2012; Shah et al., 2017). Thus, the development of a functional food containing the beneficial effect of the oat as well as the anti-inflammatory properties of the pediococcal β-glucan could have interest for the new markets. Furthermore, these β-glucan enriched cereal-based foods could be used for the elaboration of a symbiotic food in combination with one or several other probiotics.

Therefore, the β-glucan enriched matrices, generated in this work by *in situ* synthesis of the EPS by *P. parvulus* 2.6, were used to study the prebiotic and protective effects on *L. plantarum* WCFS1. Then, the evaluation of the fermentation capability of WCFS1 on both oat and rice enriched matrices showed that the viability of WCFS1 was higher in the oat matrices than in the rice matrices (Fig. 2 of Chapter 6). In addition, considering the oat matrix, there were differences between the matrix containing the bacterial β-glucan and the one not containing it, indicating that the EPS of *P. parvulus* favours the viability of *L. plantarum*. Furthermore, the matrices containing *L. plantarum* were subjected to a simulated GIT stress and a protective effect of the β-glucan on the LAB survival was observed, when the enriched oat matrix was used (Fig. 3 of Chapter 6). Thus, these cereal-based foods seem to be adequate for the administration of probiotic bacteria.

Conclussions

The results obtained in this thesis have allowed to infer the following conclusions:

1. A ELISA method for the specific detection and quantification of the bacterial 2-substituted (1,3)-β-D-glucan produced by LAB has been developed. The immune detection assay is based on a specific antibody against *S. pneumoniae* serotype 37 which produces a similar capsular β-glucan.
2. The promoter-probe vector pRCR has been developed. The plasmid carries the *mrfp* gene, that encodes the monomeric mCherry, as reporter, and it is based on the broad host range replicon of the *L. lactis* pSH71 plasmid. In this work, the usage of pRCR has been validated in three species *L. acidophilus*, *L. casei* and *L. plantarum*. In these bacteria, the fluorescence of mCherry was quantitatively determined by fluorescent spectroscopy.
3. The draft genome of *P. parvulus* 2.6 has been determined. Its size of 2.236 kpb was stablished and 2.241 genes were identified.
4. *P. parvulus* 2.6, but not its isogenic β-glucan non-producing 2.6NR strain, is able to transport and metabolise sorbitol. Moreover, 2.6 strain can use sorbitol as substrate for the production of its β-glucan.
5. The *gut* operon is located in the 39.1 kbp pPP1 plasmid of 2.6 strain, but not in the 40.0 kbp pPP1* plasmid of 2.6NR strain. The sequence analysis of both plasmids showed that they are different, and suggested that pPP1* was generated from pPP1 by DNA rearrangement.
6. The heterologous study of the *gut* operon regulation indicated that: (i) expression from P_{gut} is activated in the presence of sorbitol, (ii) glucose exerts catabolic repression on *gut* operon expression, and (iii) GutR of 2.6 acts as a transcriptional activator, while GutM is needed for the operon expression. In addition, a model has been suggested for *gut* operon regulation.
7. *P. parvulus* strains have been fluorescently labelled by transferring the pRCR12 plasmid. The labelling confers pink colour to the bacterial colonies as well as red colour to the bacteria detectable by fluorescent microscopy and spectroscopy.
8. After labelling with mCherry the 2.6 strain retained the previously observed higher binding ability to Caco-2 cells compared to that of 2.6NR. The addition of the 2.6 β-glucan to 2.6NR strain increases adhesion of this bacterium, supporting the positive influence of the polymer in binding capacity of *P. parvulus* to biotic surfaces.

9. *P. parvulus* strains were able to colonise the zebrafish GIT. The β-glucan-producing 2.6 strain had a better colonising capacity than 2.6NR. Thus, the β-glucan improved the adhesion capacity of *P. parvulus* to enterocytes in an *in vivo* zebrafish model.
10. Zebrafish larvae colonised by *P. parvulus* strains were able to survive after the *V. anguillarum* infection. Although both strains were able to counteract the effects of *Vibrio*, the better colonizing capacity of 2.6 strain provided to the zebrafish a greater protective effect against infection. Competition for the sites of attachment of the pathogen to the epithelium is presumably the mechanism by which *P. parvulus* exerts its effect.
11. The purified β-glucan of *P. parvulus* 2.6 was able to modulate the cytokine profile of zebrafish, reducing the expression levels of pro-inflammatory cytokines such as IL8 and TNFα, as well as of the protein adaptor MyD88. In a model of induced inflammation, β-glucan had an anti-inflammatory effect, inhibiting the response of neutrophils presumably through the IL8 pathway.
12. New cereal-based foods were developed by fermenting oat and rice matrices with *P. parvulus* strains. The β-glucan produced *in situ* in the matrices was detected and directly quantified with the ELISA method, without interference with the complex components of the matrices, even the naturally oat β-glucan. The prebiotic properties of the enriched matrix were validated by growth of *L. plantarum* WCFS1 in them.

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