**Characterization of four β -glucosidases acting on isoflavone-glycosides from
Bifidobacterium pseudocatenulatum IPLA 36007**

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Short title: β -glucosidases from *Bifidobacterium pseudocatenulatum*

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Abstract

Bifidobacterium pseudocatenulatum IPLA 36007 acts on isoflavone glycosides, releasing their corresponding aglycones. This strain-specific activity might be a key step in making isoflavones bioavailable and harnessing their oestrogenic activity. To investigate the molecular mechanisms involved in this activity, four glycosyl hydrolase-encoding genes in the IPLA 36007 genome (*AW18_01575*, *AW18_09810*, *AW18_08145*, and *AW18_08090*) were selected, synthesized with heterologous promoter and terminator signals (*r-β-gluA*, *r-β-gluB*, *r-β-gluD* and *r-β-gluE*, respectively), cloned into *Escherichia coli*, overexpressed as His-tagged proteins, and the enzymes purified and characterized. All four enzymes -GluA_{His}, GluB_{His}, GluD_{His} and GluE_{His}- proved to have β-glucosidase activity and deglycosylated (although at different rates) the isoflavone glycosides daidzin and genistin, releasing the corresponding aglycone moieties daidzein and genistein. GluD_{His} and GluE_{His} were also shown to hydrolyse β-glucosyl disaccharides such as cellobiose and gentiobiose, while GluA_{His} and GluB_{His} did not. Differences in activity were recorded for the four β-glucosidases at different pHs and temperatures under otherwise similar assay conditions, suggesting they have complementary activities under different environmental conditions. Two of the recombinant genes, *r-β-gluA*, and *r-β-gluD*, were cloned and expressed in the model lactic acid bacterium *Lactococcus lactis*, suggesting the β-glucosidase activity could be transferred to other starter and probiotic organisms. *B. pseudocatenulatum* IPLA 36007 contains additional β-glucosidases to those studied in this work, indicating a high level of redundancy for this enzymatic activity. Knowledge of glycoside-degrading enzymes should facilitate the development of novel, more effective or more selective prebiotics or functional foods for the promotion of

bifidobacterial numbers in the human gut. It might also be of interest in the development of novel probiotics with specific health-promoting activities.

Key words: *Bifidobacterium*; *Bifidobacterium pseudocatenulatum*; β -glucosidase; soy isoflavones; daidzein; genistein; bifidobacteria; probiotics

1. Introduction

Bifidobacterium species are among the first microbes to colonize the human gastrointestinal tract, and constitute dominant bacterial populations throughout a person's life (Odamaki et al., 2016). Bifidobacteria help maintain the health of the gut in several ways, including the competitive exclusion of pathogens, modulation of the immune system, production of essential vitamins, and provision of nutrients via the degradation of non-digestible carbohydrates (O'Callaghan and van Sinderen, 2016). These perceived health-promoting activities have led to the commercial exploitation of selected bifidobacterial strains as probiotics (Picard et al., 2005). However, fundamental knowledge related to the mechanism by which bifidobacteria contribute to host health and well-being is still scarce (Sarkar and Mandal, 2016). Such knowledge is essential in order to scientifically support their purported health benefits and their rational inclusion in functional foods.

The utilization of a wide range of carbohydrates -requiring the concerted action of uptake and degrading activities- is a key metabolic trait of bifidobacteria (de Vuyst et al., 2014). Certainly, the hydrolysis of complex dietary and host-produced carbohydrates by glycosidases would appear to be a pivotal property that allows these bacteria to colonize and survive in the gut (Pokusaeva et al., 2011). Genome analysis of

bifidobacterial strains has revealed a vast array of genes coding for these enzymes (Pokusaeva et al., 2011; Lee and O'Sullivan, 2010). Among these, β -glucosidases (which catalyse the hydrolysis of glycosidic bonds in terminal non-reducing residues of β -glucosides and oligosaccharides with the release of glucose) may play a prominent role in the probiotic action of bifidobacteria (O'Callaghan and van Sinderen, 2016). Accordingly, the bifidobacterial genomes contain many genes coding for β -glucosidases (Pokusaeva et al., 2011).

Together with gut cell-derived enzymes (Bowey et al., 2003), bacterial β -glucosidases participate in the deglycosylation of dietary β -glucoside phytoestrogens such as isoflavones, lignans, stilbenes and prenylflavonoids. This results in the formation of more bioavailable compounds, which, occasionally, can be converted afterwards into more active metabolites (Possemiers et al., 2007; Ko et al., 2014). In this sense, bifidobacteria have been shown involved in the conversion of soy isoflavone-glycosides into aglycones (Tsangalis et al., 2002; Raimondi et al., 2008), a key step in exploiting their health-associated benefits, directly through the oestrogenic activity of aglycones or by producing from them the actual active metabolites (e.g. equol) (Crozier et al., 2009). So far, however, just one β -glucosidase -from *Bifidobacterium animalis* subsp. *lactis*- with aglycone-releasing activity has been characterized at the genetic and biochemical levels (Youn et al., 2012).

To further our knowledge of these bifidobacterial enzymes, a selection of those produced by *Bifidobacterium pseudocatenulatum* IPLA 36007 were characterised. Four genes in the genome of IPLA 36007 coding for putative β -glucosidases (Alegría et al., 2014) were synthesized with heterologous promoter and terminator signals and cloned into *Escherichia coli*. The encoded enzymes were then overexpressed as histidine-tagged proteins and purified by affinity chromatography. Their basic biochemical

properties were then determined against a model substrate and their deglycosylation activities against daidzin, genistin and other substrates tested. In addition, two of these β -glucosidase-encoding genes were cloned into *Lactococcus lactis* to determine whether this bacterium could be endowed with isoflavone deglycosylating activity.

2. Material and Methods

2.1. Plasmids, bacterial strains and growth conditions

The bacterial strains and plasmid vectors used in this work are listed in Table 1. *Bifidobacterium pseudocatenulatum* IPLA 36007 was grown anaerobically at 37°C in MRS medium (Merck, Darmstadt, Germany) supplemented with 0.25% cysteine (Merck). *Escherichia coli* was grown in either Luria Bertani (LB) or in 2xTY medium at 37°C with shaking. *Lactococcus lactis* was grown (static) in GM17 (M17 supplemented with 0.5% w/v glucose) (Oxoid, Basingstoke, Hampshire, UK) at 32°C. When plates were required, agar was added at 2% (w/v). For the selection of transformants and plasmid maintenance, kanamycin (40 $\mu\text{g mL}^{-1}$) or ampicillin (100 $\mu\text{g mL}^{-1}$) was added to either liquid or solid media. 5-Bromo-4-chloro-3-indolyl- β -D-glucopyranoside (X-Glu) and isopropyl- β -D-thiogalactopyranoside (IPTG) (both from Sigma-Aldrich, St. Louis, Mo., USA) were used in different experiments at 0.004% (wt/vol) and 1 mM respectively.

2.2. Design of β -glucosidase gene cassettes for heterologous expression

The coding sequences of four putative β -glucosidases (AW18_01575, AW18_09810, AW18_08145, and AW18_08090) identified in the genome sequence of *B. pseudocatenulatum* IPLA 36007 (Alegría et al., 2014; GenBank accession no.

JEOD01000000) were synthesized (by the GenScript company, Piscataway, Nj., USA) together with the promoter sequence of the erythromycin resistance gene in pE194 from *Staphylococcus aureus* plus the terminator sequence of the *rrnB* operon of *E. coli* (Fig. 1). As a negative control, a putative α -glucosidase-encoding gene from the genome of IPLA 36007 was selected (AW18_01930), synthesised, and the protein subjected to the same analysis as the β -glucosidases. These chimeric versions of the genes were denominated r- β -*gluA* (AW18_01575), r- β -*gluB* (AW18_09810), r- α -*gluC* (AW18_01930; α -glucosidase), r- β -*gluD* (AW18_08145) and r- β -*gluE* (AW18_08090). During synthesis, HindIII and BamHI restriction enzyme sites present in the original DNA sequences were removed, while, at appropriate positions (Fig. 1), HindIII, BamHI, and XbaI sites were inserted to allow easy cloning and subcloning. These changes were made using the third-position redundancy of the genetic code without introducing amino acid changes.

Protein sequences of representative β -glucosidase encoded on the genome of several bifidobacteria species were retrieved from the GenBank database (<http://www.ncbi.nlm.nih.gov/protein/>) and compared to those from *B. pseudocatenulatum* IPLA 36007 studied in this work using the Clustal X 2.0 software (Larkin et al., 2007). A phylogenetic tree was constructed with the DNAMAN program (Lynnon, San Ramon, Ca., USA) using the neighbour-joining algorithm.

2.3. Cloning and expression of recombinant glucosidase genes in *E. coli*

The synthesized glucosidase genes were independently cloned into pUC57 by directional cloning, either using the corresponding restriction enzyme sites or employing the In-Fusion kit (Clontech, Mountain View, Ca., USA) following the manufacturer's instructions. Electrocompetent *E. coli* DH10B cells were prepared as

reported elsewhere (Sambrook and Russell, 2001) and transformed with the ligation mixtures in a Gene Pulser apparatus (Bio-Rad, Richmond, Ca., USA). Transformants with β -glucosidase activity were revealed by transferring colonies to 2xTY agar plates containing ampicillin, IPTG, and the chromogenic substrate X-Glu. Plasmid DNA of positive colonies showing blue colour on the plates was purified by using the QIAprep Spin Miniprep kit (Qiagen, Valencia, Ca., USA). Constructs were verified by restriction endonuclease digestion and sequencing.

2.4. Glucosidase activity in *E. coli* cell-free extracts

The β -glucosidase activity of the constructs was measured in cell-free extracts using *p*-nitrophenyl(*p*NP)- β -D-glucopyranoside (*p*NP- β -Glu) (Sigma-Aldrich) as a substrate. The reaction mixture was composed of 800 μ L of 40 mM sodium acetate buffer (pH 5.5), 100 μ L of 10 mM of *p*NP- β -Glu or *p*NP- α -Glu, and 100 μ L of each of the cell-free extracts. After 30 min at 37°C the reaction was stopped by adding 1 mL of cold 1 M sodium carbonate, and the solution clarified by centrifugation at 19,000 $\times$ *g* for 10 min at 4°C. The *p*NP released was determined spectrophotometrically, measuring the increase in absorbance at 410 nm against a calibration curve of *p*NP (Sigma-Aldrich). α -Glucosidase activity was measured in a similar manner by using as a substrate *p*NP- α -D-glucopyranoside (Sigma-Aldrich).

2.5. Daidzin deglycosylation activity of *E. coli* cell-free extracts

Overnight cultures were pelleted, suspended in 0.1 M phosphate buffer (pH 6.8) containing 100 μ M daidzin (LC Labs, Woburn, Ma., USA) and incubated at 37°C for 4 h. The isoflavone aglycones were then extracted following the procedure of Guadamuro et al. (2015) with modifications. Briefly, samples were centrifuged at 800 rpm for 10

min; 3 mL of each supernatant were then collected and their isoflavone contents extracted using Bond Elut-C18 solid-phase cartridges (Agilent, Santa Clara, Ca., USA). After drying, the organic extracts were dissolved in 100 µl of HPLC grade methanol. A 10 µL aliquot of the sample was then loaded onto 60 F₂₅₄ thin layer chromatography (TLC) silica gel plates (Merck), developed in a vertical developing chamber with a 2:1 toluene:acetone (both from Merck) solvent system, and revealed under UV light (256 nm).

2.6. Cloning of the constructs in an overexpression system

To overexpress the β-glucosidases as His-tagged proteins from the chimeric glucosidase genes, plasmid DNA of the constructs in pUC57 was purified using the High Pure Plasmid Isolation kit (Roche, Basel, Switzerland), digested with the restriction enzymes NheI and BamHI, and ligated with T₄ DNA ligase (Roche) to double digested pET28a(+) (Novagen, San Diego, Ca., USA). The ligation mixtures were then electrotransformed into *E. coli* DH10B. Constructs in pET28a(+) were verified by digestion with restriction enzymes and sequencing. Finally, DNA of the constructs in pET28a(+) was purified as above and transformed into the overexpression host *E. coli* BL21(DE3) (Novagen).

2.7. Purification of recombinant proteins

Selected transformants of *E. coli* BL21(DE3) containing each of the pET28a(+) constructs (Table 1) were incubated overnight with agitation at 37°C in LB broth supplemented with 25 µg mL⁻¹ kanamycin. The cultures were then re-inoculated into 300 mL of fresh medium, grown until mid-exponential phase (0.5 absorbance at 600 nm), and induced for r-*glu* overexpression by the addition of 1 mM IPTG. The cells

were then incubated at 37°C for 20 h, harvested by centrifugation, suspended in lysis buffer (50 mM NaH₂PO₄, 300 mM NaCl, 10 mM imidazole), and disrupted at 2.3 bars in a FastPrep cell disruptor (Constant Systems, Daventry, UK). Recombinant glucosidases were purified at 4°C using Ni-nitrilotriacetic acid affinity chromatography as recommended by the supplier of the latter compound (Qiagen). Purified proteins were electrophoresed under denaturing conditions in 6% sodium dodecyl sulphate-polyacrylamide gels (SDS-PAGE) in a mini-protean apparatus (Bio-Rad) and visualized with Coomassie Brilliant Blue R-250 (Bio-Rad).

The β-glucosidase activity of the purified proteins was measured using *p*NP-β-Glu as a substrate as described above for the cell-free extracts. Protein concentration was determined by the Bradford method using the BCA Protein Assay kit (Pierce, Rockford, IL, USA), employing bovine serum albumin as a standard. One unit of activity was defined as the amount that releases 1 μmol of *p*NP per min.

2.8. Biochemical characterization of the purified enzymes

To examine dependence on pH, the activity of the purified enzymes was measured against the substrate *p*NP-β-Glu between pH 3.0 and 8.0 (achieved using citrate or phosphate buffers) and recording the *p*NP produced. Temperature dependency was measured using the same substrate over the range 10-90°C. The kinetics of hydrolysis was also determined using *p*NP-β-Glu (0.1-8.0 mM), after verification of the reaction rate linearity, by stopping reactions at 30 min and measuring the *p*NP released as above. Additionally, substrate specificity was evaluated using the following *p*NP-derivatives: *p*NP-β-D-galactopyranoside (*p*NP-β-Gal), *p*NP-β-D-arabinopyranoside (*p*NP-β-Ara) and *p*NP-α-D-glucopyranoside (*p*NP-α-Glu) (all from Sigma-Aldrich). Purified enzymes were also assayed against maltose, gentiobiose, melibiose, cellobiose and

isomaltose (all from Sigma-Aldrich, except maltose from Merck) (2 μ g purified enzyme, 10 mM of each substrate sugar in 40 mM sodium acetate buffer [pH 5.5]) overnight at 37°C. Sugar hydrolysis was also investigated by TLC. One microliter of each reaction was loaded onto TLC plates and developed in a vertical chamber using a 2:1:1, ethyl acetate, acetic acid and water solvent system. Spots were revealed by heating at 120°C after spraying with 5% sulphuric acid in ethanol.

The deglycosylation of daidzin and genistin was performed in 50 mM phosphate buffer (pH 6.0) with 9 μ g of purified r-Glu and 750 μ M daidzin- or genistin-7-O- β -D-glucoside (both from Sigma-Aldrich) in a final volume of 65 μ L. After overnight incubation at 37°C, the mixture was heated at 95°C for 5 min and cooled on ice. Reactions were visualized by TLC. For this, 10 μ L of each reaction was loaded onto the TLC plates, and developed and revealed as above.

2.9. Cloning and expression of r- β -glu genes in *Lactococcus lactis* MG1363

Construction of a recombinant *Lactococcus lactis* MG1363 strain expressing r- β -glu genes was achieved using the In-Fusion system as above. Briefly, r- β -glu genes were PCR amplified from the constructs in pUC57 using primers P21/22-F and P21/22-R (Table 1) in order to add to the amplicons 20 bp sequences that were identical to the extremes of EcoRI linearized vector p21/22 (Sánchez and Mayo, 2004). After the In-Fusion reaction, *L. lactis* MG1363 was electrotransformed with the mixture and transformants selected onto GM17 agar plates containing erythromycin (5 μ g mL⁻¹) and X-Glu. Plasmid DNA was isolated from randomly selected blue colonies by a modification of the procedure of O'Sullivan and Klaenhammer (1993), involving the addition of 4 μ L of mutanolysin (5 U mL⁻¹) and 20 μ L of proteinase K (20 mg mL⁻¹) to the lysis buffer before phenol/chloroform extraction. Constructs were verified by

digestion with restriction enzymes.

3. Results

3.1. Cloning, expression and overexpression of glycosidase genes in *E. coli*

Five ORFs from the genome of *B. pseudocatenulatum* IPLA 36007, encoding putative β -glucosidases (AW18_01575, AW18_09810, AW18_08145, and AW18_08090) or α -glucosidases (AW18_01930), were synthesized (r- β -gluA, r- β -gluB, r- α -gluC, r- β -gluD and r- β -gluE, respectively) under the control of synthetic heterologous promoter and terminator sequences, following the general outline shown in Figure 1.

The synthetic genes were cloned in pUC57, and glucosidase-active clones in *E. coli* retrieved from agar plates containing chromogenic substrates. Positive transformants were obtained for all genes. The r- α -gluC clone and the α -glucosidase it encoded were used as β -glucosidase negative controls in the majority of subsequent experiments. TLC analysis of the reactions between isoflavone-glycosides and *E. coli* cell-free extracts revealed all clones (except, as expected r- α -gluC) to produce daidzein from daidzin (Supplementary Fig. 1).

For protein overexpression, DNA of all pUC-derived clones was digested with the restriction enzymes NheI and BamHI and cloned into double digested pET28a(+). The new constructs were obtained and verified in *E. coli* DH10B. After the transfer of the clones to *E. coli* BL21(DE3), cultures carrying pET28a(+)-derivatives were induced with IPTG and the overexpressed proteins purified to SDS-PAGE homogeneity by Ni-nitrilotriacetic acid affinity chromatography. By way of example, Supplementary Fig. 2 shows an SDS-PAGE gel of the purification steps for GluB_{His} β -glucosidase. The molecular mass of the purified enzymes agree well with the calculated molecular mass

of their respective gene product. The purification yields for GluA_{His}, GluB_{His}, GluC_{His}, GluD_{His} and GluE_{His} were 0.80, 3.29, 6.39, 3.54, 4.32 mg L⁻¹ of culture respectively. The low purification yield of GluA_{His} might result from toxicity of the overexpression of this protein in *E. coli*, as confirmed by the selective counting of colonies before and after IPTG induction (data not shown). Introducing the pET28a(+)-r-*β-gluA* construct into *E. coli* BL21(DE3)-pLysS in an attempt to improve the purification of GluA_{His} by avoiding the leaky expression of this *β*-glucosidase was unsuccessful.

3.2. Characterization of purified *β*-glucosidases

As expected, the purified GluA_{His}, GluD_{His} and GluE_{His} showed activity against the chromogenic substrate *pNP-β-Glu* with a specific activity of 1.74, 390.18 and 219.95 μ M/mg/min respectively. The low activity of GluA_{His} against *pNP-β-Glu* might be explained by the low yield, as reported above, and the concomitant presence in the eluate of several protein bands without *β*-glucosidase activity (which might be degradation products of this toxic enzyme; data not shown). Surprisingly, the GluB_{His} enzyme showed no activity against any of the *pNP* derivatives analysed. As anticipated, GluC_{His} showed activity with *pNP-α-Glu* but not towards *pNP-β-Glu*.

As revealed by TLC, all four *β*-glucosidases were active against the isoflavone glycosides daidzin and genistin, releasing the aglycones daidzein and genistein respectively (Fig. 2). Though the products of the reactions were not quantified, GluA_{His} and GluD_{His} seemed to produce more aglycones than GluB_{His} and GluE_{His} under the same assay conditions. The TLC technique was also used to examine the activity of the purified enzymes against several *α*- and *β*-glucoside sugars. GluD_{His} and GluE_{His} hydrolysed *β*-glucosides such as cellobiose (O-*β*-D-glucosyl-[1→4]-D-glucose) and gentiobiose (O-*β*-D-glucosyl-[1→6]-D-glucose), but, as expected for a *β*-glucosidase,

not against α -glucosides such as maltose (O- α -D-glucosyl-[1 $\rightarrow$ 4]-D-glucose), isomaltose (O- α -D-glucosyl-[1 $\rightarrow$ 6]- α -D-glucose) or melibiose (O- α -D-galactosyl-[1 $\rightarrow$ 6]-D-glucose). In contrast, GluA_{His}, GluB_{His} degraded none of these disaccharides (Supplementary Fig. 3).

The maximum relative activity against *p*NP- β -Glu of GluA_{His}, GluD_{His} and GluE_{His} laid between pH 5.0 and 6.5. GluA_{His} had an optimum pH of around 6.5; that of GluD_{His} and GluE_{His} was pH 5.5. GluA_{His} showed an optimum temperature of 30-35°C, with relatively strong activity between 20 and 50°C. GluD_{His} and GluE_{His} showed peaks of activity at 40 and 45°C respectively, declining sharply at lower and higher temperatures (Fig. 3). The V_{max} for GluD_{His} and GluE_{His} was 75.26 and 10.10 μ M min⁻¹ respectively; the K_m was 0.69 and 10.78 mM respectively. For technical reasons, the kinetics of GluA_{His} (low protein concentration) and GluB_{His} (lack of activity against *p*NP- β -Glu) could not be determined.

3.3. Heterologous expression in *Lactococcus lactis*

To provide proof of concept that the functional expression of isoflavone-deglycosylation activity can be transferred to a lactic acid bacterium (LAB), r- β -gluA and r- β -gluD were selected for cloning and expression in *Lactococcus lactis*. Plasmids of the corresponding constructs in pUC57 were digested with HindIII and XbaI and cloned into the *E. coli*-LAB shuttle vector p21/22. The new constructs (Table 1) were obtained in *E. coli* DH10B and then transformed into *L. lactis* MG1363 by electroporation. The β -glucosidase activity of *L. lactis* cells harbouring p21/22-r- β -gluA and p21/22-r- β -gluD on *p*NP- β -Glu was respectively 5 and 76 times greater than that of the controls. TLC analysis showed that both transformants could convert daidzin into daidzein (Supplementary Fig. 4).

4. Discussion

In this work, five genes from the genome of *B. pseudocatenulatum* IPLA 36007 thought to encode α - and β -glucosidases were cloned into *E. coli*, overexpressed, and their proteins purified and characterized. Though not well studied, evidence is accumulating for direct (Trindale et al., 2003) and reverse (Parche et al., 2006) regulatory carbon catabolite repression (CCR) mechanisms in bifidobacteria. In fact, open reading frames encoding LacI-like regulatory proteins have been seen in the vicinity of most glycosidase-encoding genes (Alegría et al., 2014). To avoid regulatory carbon catabolite repression (CCR) interference during cloning and expression, the native promoter and terminator sequences of the selected genes were replaced during synthesis by heterologous signals. In this work, the promoter of the erythromycin resistance gene of pE194 from *S. aureus* and the ρ -independent terminator of the *rrnB* operon from *E. coli* were used as signals for heterologous expression of the glucosidase genes. These signals have already been shown to be recognized by Gram-positive and Gram-negative bacteria (Schwendener and Perreten, 2015; Horinouchi and Weisblum, 1982).

Compared to α -glucosidases (encoded by the so called *agl* genes in the literature) (Kelly et al., 2016; Pokusaeva et al., 2011; van den Broek et al., 2008), the β -glucosidases of bifidobacteria have been poorly studied. β -Glucosidases cleave β -D-glycosidic linkages, liberating glucose moieties from phytoestrogen-glycosides (del Rio et al., 2013). As such, β -glucosidase activity in bifidobacteria has been associated with the conversion of sennosides from senna and rhubarb to sennidins (Yang et al., 1996), the activation of the phytotoxin cycasin into a mutagenic compound (Choi et al., 1996),

and the hydrolysis of isoflavone-glycosides into aglycones (Donkor and Shah, 2008; Raimondi et al., 2008; Reckha and Vijayalakshmi, 2011). The latter activity is thought to be pivotal in the absorption and subsequent metabolism of soy isoflavones, the consumption and adequate metabolism of which are considered beneficial to human health (Mayo et al., 2016).

The β -glucosidases of *B. pseudocatenulatum* IPLA 36007 characterized here are part of a vast array of genes within its genome that code for carbohydrate-modifying enzymes. Of these, 52 genes might encode different glycosyl hydrolases, and between seven and nine have already been considered that may code for β -glucosidases (Alegría et al., 2014). All four β -glucosidases characterized in this work were shown to deglycosylate the isoflavone-glycosides daidzin and genistin, releasing the aglycone moieties daidzein and genistein respectively (Fig. 2). However, although the enzymatic redundancy for deglycosylation in IPLA 36007 is here shown to be large, additional β -glucosidases from this strain may further act on soy isoflavones. All four enzymes showed differences in activity at different pHs and temperatures under otherwise similar conditions (as shown for two of them in Fig. 3), which suggests they might have complementary activities under distinct environmental conditions. However, the differential efficiency in transforming the isoflavone-glycosides into aglycones by the different enzymes could not be quantified. The K_m and V_{max} of some enzymes might be measurable using *p*NP- β -Glu, but the poor solubility of isoflavone-glycosides in water and the obligate need of using organic solvents that can otherwise be detrimental for enzyme activity, hindered the collection of results for some others.

The isoflavone-glycoside deglycosylation activity of bifidobacterial whole cells (i.e., not pure enzymes) varies widely between species and strains (Raimondi et al., 2009; Wei et al., 2007; Tsangalis et al., 2002). Genomes of bifidobacteria are plenty of

glucosidase-encoding genes (Milani et al., 2015), of which many are β -glucosidases. Indeed, genome-sequenced bifidobacterial strains contain between two (*Bifidobacterium bifidum* S17) and 17 (*Bifidobacterium dentium* Bd1) genes encoding putative β -glucosidase (for a recent review see O'Callahan and van Sinderen, 2016), which mostly fall within the CAZy GH families 1, 3 and 5 (<http://www.cazy.org/>). Orthologous genes encoding β -glucosidases related to those characterized in this work were shown to be present in representative genomes of several bifidobacteria species (Fig. 4). However, as shown in the figure, the enzymes characterized in this study showed low homology to those reported in *B. animalis* subsp. *lactis* SH5, the single strain in which isoflavone deglycosylation activity has already been characterized (Youn et al., 2012). In addition to the number of genes, substrate specificity (as it happens for the different enzymes of this study) might also account for the deglycosylating differences among species and strains. None of the deduced proteins in the present study have shown a signal peptide-like sequence that might drive secretion (suggesting the enzymes to be all intracellular) (Alegría et al., 2014). Thus variations in activity might further be owed to differences in the ability to transport isoflavone-glycosides into the cells. However, the expression of two β -glucosidase genes in *L. lactis* accompanied by the acquisition of deglycosylation phenotype argues against the need for specialized transport.

In conclusion, four β -glucosidases from *B. pseudocatenulatum* were cloned in *E. coli*, overexpressed, purified and biochemically characterized. Improved knowledge of glycoside-degrading enzymes should facilitate the development of novel, more effective or more selective prebiotic compounds to specifically enhance numbers of bifidobacteria species metabolizing them as a mean to promote health in the human gut in way similar to the current use of oligosaccharides and inulin (Oliveira et al., 2012). It should also help to design and use of novel or renew probiotics, based on their,

scientifically-sound, health-related activities, such as the transformation of isoflavone-glycosides into aglycones. Probiotic strains having definite health benefits with well-known underlying operating mechanisms should replace currently-in-use “all purpose” probiotics. Finally, it could further help in the transfer of enzyme-associated functionality to technologically-relevant starter and probiotic strains. The heterologous expression of β -glucosidase genes from *B. pseudocatenulatum* in *L. lactis* confirmed the possibility of using recombinant LAB strains for the production of aglycone-enriched foods.

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Authors Contribution Statement

BM designed the study, provided material and human resources, contributed to discussion of the results, and wrote the manuscript. ABF, LG, AA, and LV conducted the experiments, contributed to the discussion, revised the text and approved the final version of the manuscript.

Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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540 **Table 1.-** Bacterial strains, plasmids and primers used in this study.

Strain, plasmid, or primer	Relevant genotype, description or sequence (5'-3')	Source or reference
Bacteria		
<i>Bifidobacterium pseudocatenulatum</i> IPLA 36007	Human intestinal isolate with deglycosylation activity against soy isoflavone-glycosides	Delgado et al. (2008)
<i>Escherichia coli</i> DH10B	$\Delta(lac)X74$, $\phi 80lacZ\Delta M15$, $araD139$, $\Delta(ara, leu)7697$, $mcrA$, $\Delta(mrr-hsdRMS-mcrBC)$, Str^R , λ^-	Invitrogen
<i>E. coli</i> BL21(DE3)	F ⁻ , <i>ompT</i> , <i>gal</i> , <i>dcm</i> , <i>lon</i> , <i>hsdSB(rB-mB-)</i> , $\lambda(DE3[lacI, lacUV5-T7\ gene\ 1, ind1, sam7, nin5])$	Novagen
<i>E. coli</i> BL21(DE3)-pLysS	<i>E. coli</i> BL21(DE3) carrying plasmid pLysS, which harbours a lysozyme gene under the control of the T7 promoter	Novagen
<i>Lactococcus lactis</i> MG1363	Plasmid-free derivative of <i>L. lactis</i> SH4109	Gasson (1983)
Plasmids		
pUC57	Cloning vector, Am ^r	Genscript
pUC57-r- β - <i>gluA</i>	ORF AW18_01575 from the <i>B. pseudocatenulatum</i> IPLA 36007 genome cloned in pUC57 under promoter and terminator signals of the erythromycin resistance gene of pE194 (<i>Staphylococcus aureus</i>) and the <i>rrnB</i> operon (<i>E. coli</i>), respectively	This work
pUC57-r- β - <i>gluB</i>	The same for ORF AW18_09810	This work
pUC57-r- α - <i>gluC</i>	The same for ORF AW18_01930	This work
pUC57-r- β - <i>gluD</i>	The same for ORF AW18_08145	This work
pUC57-r- β - <i>gluE</i>	The same for ORF AW18_08090	This work
pET28a(+)	Expression vector under the T7 expression region carrying N- and C-His-Tag sequences, Km ^r , 5,369 bp	Novagen
pET28a(+)-r- β - <i>gluA</i>	ORF AW18_01575 cloned in pET28a(+)	This work
pET28a(+)-r- β - <i>gluB</i>	ORF AW18_09810 cloned in pET28a(+)	This work
pET28a(+)-r- α - <i>gluC</i>	ORF AW18_01930 cloned in pET28a(+)	This work
pET28a(+)-r- β - <i>gluD</i>	ORF AW18_08145 cloned in pET28a(+)	This work
pET28a(+)-r- β - <i>gluE</i>	ORF AW18_08090 cloned in pET28a(+)	This work
p21/22	An <i>E. coli</i> - <i>L. lactis</i> shuttle vector derived from the pBM02 plasmid of <i>L. lactis</i> subsp. <i>cremoris</i> P8-2-47	Sánchez and Mayo (2004)
p21/22-r- β - <i>gluA</i>	ORF AW18_01575 and promoter and terminator sequences in pUC57 cloned in p21/22	This work
p21/22-r- β - <i>gluD</i>	ORF AW18_08145 and promoter and terminator sequences in pUC57 cloned in p21/22	This work
Oligonucleotide primers		
P21/22-F	GTAAAACGACGGCCAGTGAAGCTTCGAAATGATACA CCAATCAG	This work
P21/22-R	GATCGGGTACCGAGCTCGTCTAGAGGATCCCAAACA ACAGATAAAACG	This work

Am^r, Kmr, and Em^r, resistance to ampicillin, kanamycin and erythromycin, respectively.

P

AAGCTTCGAAATGATACACCAATCAGTGCAAAAAAAGATATAATGGGAGATAAGACGGT

HindIII

TCGTGTTTCGTGCTGACTTGCACCATATCATAAAAAATCGAAACAGCAAAGAATGGCGGAA

-35

ACGTAAAAGAAGTTATGGAAATAAGACTTAGAAGCAAACCTTAAGAGTGTGTTGATAGTG

-10

CAGTATCTTAAATTTTGTTATAATAGGAATTGAAGTTAAATTAGATGCTAAAAATTTGT

RBS

AATTAAGAAGGAGTGCTAGC**atg**

NheI SC

β -glucosidase gene

T

SC

taaGCATCAAATAAAACGAAAGGCTCAGTCGAAAGACTGGGCCTTTCGTTTTATCTGTT

GTTTGGGATCCTCTAGA

BamHI-XbaI

Figure 1

A

B

Figure 2

Figure 3

Figure 4

Figure Captions

Figure 1.- General strategy of designing the synthetic β -glucosidase-encoding genes for cloning and expression in *Escherichia coli* DH10B. P is the promoter of the erythromycin resistance gene of pE194 from *Staphylococcus aureus* and T is the terminator of the *rrnB* operon from *E. coli*.

Figure 2.- TLC showing deglycosylation activity of purified β -glucosidases from *B. pseudocatenulatum* IPLA 36007 on daidzin (A) and genistin (B). Lanes 1, 2, 3, 4, 5 and 6, daidzin or genistin, after overnight incubation at 37 °C without enzyme, and with the crude extract of GluA_{His} and the purified enzymes of GluB_{His}, GluC_{His}, GluD_{His}, and GluE_{His}, respectively. Lanes 7, daidzin and genistin markers, respectively. Lanes 8, daidzein and genistein markers, respectively.

Figure 3.- Effects of pH and temperature on the activity of the purified β -glucosidases GluA_{His}, GluD_{His} and GluE_{His} from *B. pseudocatenulatum* IPLA 36007. Experiments were performed in triplicate. Activity of each enzyme at the optimum pH and temperature was defined as 100%.

Figure 4.- Phylogenetic tree of different β -glucosidases present in the genome of sequenced strains from several bifidobacterial species (left) and amino acid sequence identity to those of *B. pseudocatenulatum* IPLA 36007 (right). Similar percentages of identity between the proteins are coded with the same colour.

Supplementary Figure 1.- TLC showing deglycosylation of daidzin by *Escherichia coli* DH10B strain transformed with r- β -gluA, r- β -gluB, r- α -gluC, r- β -gluD and r- β -gluE genes from *B. pseudocatenulatum* IPLA 36007 cloned on the pUC57 vector on lanes 1, 2, 3, 4, 5, respectively. Lane 6 medium containing daidzin inoculated with *E. coli* DH10B cells harbouring pUC57 as a negative control. Lanes 7 and 8 daidzin and daidzein markers, respectively.

Supplementary Figure 2.- SDS-PAGE of purified fractions of the β -glucosidase GluB_{His} during overexpression in *Escherichia coli* BL21(DE3). Order key: Lane 1, cell lysate of the expression host *E. coli* BL21(DE3); Lane 2, cell lysate of *E. coli* BL21(DE3) harbouring the cloning vector pET28a(+); Lane 3, cell lysate of *E. coli* BL21(DE3) harbouring pET28a(+)-r- β -gluB (overloaded); Lane 4, cell lysate of *E. coli* BL21(DE3) harbouring pET28a(+)-r- β -gluB after induction with 1 mM IPTG; Lane 5, washing eluate of the Ni-NTA column with 20 mM imidazole; Lanes 6, 7 and 8, consecutive elutions of GluB_{His} from the Ni-NTA column with 250 mM imidazole. MW, protein molecular size marker; numbers indicate the size of the markers in kDa.

A

B

Supplementary Figure 3.- Glycosidase activity of purified β -glucosidases on different sugar substrates. Order key: Panel A, Lanes 1 to 5 and lanes 6 to 10, activity of GluA_{His} and GluB_{His}, respectively on melibiose, gentiobiose, isomaltose, cellobiose, and maltose, respectively. Panel B, lanes 1 to 5 and 6 to 10, activity of GluD_{His} and GluE_{His}, respectively on gentiobiose, cellobiose, melibiose, isomaltose and maltose, respectively. In both panels, lanes G and F, contain glucose and fructose markers, respectively.

Supplementary Figure 4.- TLC showing deglycosylation activity on daidzin of cell-free extracts of *Lactococcus lactis* MG1363 expressing recombinant *Bifidobacterium pseudocatenulatum* IPLA 36007 β -glucosidases GluA and GluD. Order key: Lane 1, *L. lactis* MG1363 transformed with the empty p21/22 vector. Lanes 2 and 3, transformants carrying r- β -gluA and r- β -gluD genes, respectively. Lanes 4 and 5 daidzin and daidzein markers, respectively.