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Influence of different lignan compounds on enterolignan production by *Bifidobacterium* and *Lactobacillus* strains

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ABSTRACT

Enterolignans, i.e. enterodiol and enterolactone, are polyphenols derived from the microbial metabolism of dietary lignans. They are considered phytoestrogens because of their estrogenic/antiestrogenic activity, which confers them benefits to human health when they reach sufficient levels in plasma. Hence, there is a great interest in studying the bacteria involved in enterolignan production. In the present study, three bifidobacterial strains (*Bifidobacterium bifidum* INIA P466, *Bifidobacterium catenulatum* INIA P732 and *Bifidobacterium pseudolongum* INIA P2) were found capable of producing low levels of enterodiol (2–11 μM) from lignan extracts; while another one (*Bifidobacterium pseudocatenulatum* INIA P946) was found to produce an important increment of the lignan secoisolariciresinol (SECO). Subsequently, the three enterodiol-producing bifidobacteria and another three *Lactobacillus* strains previously identified as enterolignans producers (*Lactobacillus gasseri* INIA P508, *Lactobacillus salivarius* INIA P448 and *Lb. salivarius* INIA P183), were tested on pure lignans yielding both enterodiol and enterolactone from secoisolariciresinol (SECO), while they did not metabolised the other lignan tested (i.e. matairesinol). *B. catenulatum* INIA P732 and *Lb. gasseri* INIA P508 were the strains that transformed the greatest percentage of SECO, yielding enterolactone concentrations above 2 mM. In addition, the formation of the intermediate compound dihydroxyenterodiol was observed as part of SECO transformation by all the strains. In this work, we have demonstrated for the first time how strains of *Bifidobacterium* and *Lactobacillus* are capable of carrying out the complete enterolignan metabolism, transforming a purified lignan (SECO) into enterodiol and enterolactone. The isolation and characterization of bacteria able to metabolize lignans and produce enterolignans, especially belonging to *Bifidobacterium* and *Lactobacillus* genera, is of biotechnological interest, because of their potential application in functional foods and as probiotics.

1. Introduction

Phytoestrogens are polyphenols found in plants or derived from plant precursors (Milder et al., 2005; Valsta et al., 2003), which have similarities in chemical structure to mammalian estrogens (Farooq, 2015). Five kinds of phytoestrogens can be found, namely isoflavones, ellagitaninns, lignans, stilbenes and coumestans (Landete et al., 2016). Lignans are the most abundant phytoestrogens in the Western diet, as they are present in high concentrations in oilseeds (flax, soy, grapeseed, sesame) and whole grain cereals and flours, as well as in some fruits and vegetables, coffee, tea and wine (Durazzo et al., 2013; Landete, 2012). Lignans such as secoisolariciresinol (SECO) and its precursor secoisolariciresinol diglucoside (SDG) are the most abundant lignans found in the diet. In addition, other lignans such as matairesinol (MAT), and the SECO precursors pinoresinol and lariciresinol, can also be found in some plant foods (Landete, 2012; Milder et al., 2005).

Dietary lignans have limited biological properties because of their low bioavailability (Landete et al., 2016; Quartieri et al., 2016) and must be converted by the intestinal microbiota into enterolignans, i.e. enterodiol (END) and enterolactone (ENL), which are much more bioavailable than their lignan precursors (Clavel et al., 2006a; Clavel et al., 2005). Enterolignans exert estrogen agonism and antagonism (Mueller et al., 2004), show enzyme-inhibiting properties and have antioxidant activities higher than dietary lignans (Kitts et al., 1999). In this way, the presence of sufficient concentrations of END and ENL in human plasma have been related with a lower incidence of breast and other types of cancer and cardiovascular disease (Aarestrup et al., 2013; Piller et al., 2006; Seibold et al., 2014; Vanharanta et al., 1999; Yoder

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et al., 2015), while the intake of a lignan-rich diet has not shown so evident association.

Enterolignan production by intestinal microbiota is quite extended among individuals, although there is a variability in the amounts produced (Gaya et al., 2016b), which could be attributed to differences in the microbiota composition. To date, scientific publications have reported that plant lignans can be converted by a consortium of intestinal bacteria into END and ENL (Wang et al., 2010; Woting et al., 2010; Xie et al., 2003). *Eggerthella lenta* is able to dehydroxylate dihydroxyenterodiol (DHEND) to produce END (Clavel et al., 2006b; Jin et al., 2007b); and several clostridia and *Ruminococcus* spp. strains have been described in the dehydrogenation of END into ENL (Clavel et al., 2006b; Clavel et al., 2007; Jin et al., 2007a). Recently, we described the obtaining of enterolignans from flax extracts by the action of sole strains, specifically *Bifidobacterium adolescentis* INIA P784 able to produce END (Gaya et al., 2017a) and *Lactobacillus gasseri* and *Lactobacillus salivarius* strains able to produce both END and ENL (Bravo et al., 2017).

In the present work we extended the study of the enterolignan production from lignan extracts to other strains of bifidobacteria, together with the analysis of enterolignan metabolism from pure lignans (SECO and MAT) by the selected *Bifidobacterium* and *Lactobacillus* strains. Given that lignans are the most common phytoestrogens in Western diet, the health effects of enterolignans and the interindividual variability of their production, it is of interest the characterization of bacteria able to transform dietary lignans into enterolignans. That would ensure a sufficient input of enterolignans to the organism, either by means of functional foods or as probiotics.

2. Material and methods

2.1. Bacterial strains and growth conditions

Bifidobacterium and *Lactobacillus* strains of human origin tested in this work are listed in Table 1. Bifidobacteria were cultivated under strict anaerobic conditions at 37 °C in RCM broth (BD, Le Pont de Claix, France). Lactobacilli were grown under strict anaerobic conditions at 37 °C in MRS (Oxoid Ltd., Basingstoke, United Kingdom).

2.2. Chemicals and solvents

The HPLC grade standard compounds SECO, MAT, END and ENL were purchased from Merck KGaA (Darmstadt, Germany). Methanol, acetic acid and acetonitrile were of HPLC grade (LabScan, Gliwice, Poland). LinumLife EXTRA, a lignan extract from flax seeds, were provided by Frutarom Netherlands BV (Veenendaal, the Netherlands).

2.3. Screening of Bifidobacterium strains producing enterolignans

Bifidobacterium strains were inoculated 0.1% in 10 mL of BHI medium (Biolife, Milan, Italy) containing 0.5 g/L of L-cysteine (Merck KGaA), sterilized at 121 °C for 20 min, and supplemented with flax extracts (2 g/L) dissolved in DMSO (Merck KGaA). BHI has been used in previous work for the analysis of the production of enterolignans and other phytoestrogens by isolated bacteria (Bravo et al., 2017) and by intestinal microbiota (Gaya et al., 2016b). The pH of the broth was adjusted to 7.4 with NaOH. When present, the amount of DMSO in the broth did not affect bacterial growth. *Bifidobacterium* strains with the polyphenols were incubated at 37 °C for 5 days under anaerobic conditions in sealed jars (Oxoid Ltd.) using AnaeroGen sachets (Oxoid Ltd.).

BHI broth with flax extracts without bacteria was used as negative control. All experiments were done in duplicate and the results were reproducible.

2.4. Analysis of enterolignan production from pure lignan compounds

The metabolism of SECO and MAT was analysed in the *Bifidobacterium* strains that were able to produce END from flax extract, and in three *Lactobacillus* strains that had previously showed END and ENL production from lignan extracts (Bravo et al., 2017). Besides them, two *Bifidobacterium* strains unable to produce enterolignans from flax extract were also tested. The strains were inoculated 0.1% in 10 ml of BHI medium containing 0.5 g/L of L-cysteine and SECO (5 mM) or MAT (5 mM). The pH of the broth was adjusted to 7.4 with NaOH. The inoculated broths supplemented with flax extract and pure compounds were incubated at 37 °C for 5 days under anaerobic conditions. Non inoculated BHI broths with SECO and MAT were used as controls. Additionally, two strains that had not produced END from lignan extract were also incubated with the pure compounds, to serve as negative controls All experiments were done in duplicate and the results were reproducible.

Table 1
Bacterial strains tested in this work.

Strain	Origin	Reference
B. adolescentis INIA P549	Breast-fed infant faeces	Rodríguez et al., 2012
B. animalis INIA P490	Breast-fed infant faeces	Rodríguez et al., 2012
B. animalis INIA P913	Adult faeces	INIA culture collection
B. bifidum INIA P466	Breast-fed infant faeces	Rodríguez et al., 2012
B. breve INIA P12	Breast-fed infant faeces	Rodríguez et al., 2012
B. breve INIA P367	Breast-fed infant faeces	Rodríguez et al., 2012
B. breve INIA P763	breast-fed infant faeces	Peirotén et al., 2018
B. breve INIA P776	Child faeces	Peirotén et al., 2018
B. catenulatum INIA P732	Breast-fed infant faeces	Peirotén et al., 2018
B. catenulatum INIA P765	Adult faeces	Peirotén et al., 2018
B. catenulatum INIA P825	Child faeces	Peirotén et al., 2018
B. dentium INIA P883	Adult faeces	INIA culture collection
B. infantis INIA P593	Breast-fed infant faeces	Rodríguez et al., 2012
B. infantis INIA P722	Breast-fed infant faeces	Peirotén et al., 2018
B. infantis INIA P728	Breast-fed infant faeces	Peirotén et al., 2018
B. longum INIA P9	Breast-fed infant faeces	Rodríguez et al., 2012
B. longum INIA P750	Breast-fed infant faeces	Peirotén et al., 2018
B. pseudocatenulatum INIA P846	Child faeces	Peirotén et al., 2018
B. pseudocatenulatum INIA P946	Breast-fed infant faeces	INIA culture collection
B. pseudolongum INIA P2	Breast-fed infant faeces	Rodríguez et al., 2012
Lb. gasseri INIA P508	Breast-fed infant faeces	Rodríguez et al., 2012
Lb. salivarius INIA P448	Breast-fed infant faeces	Rodríguez et al., 2012
Lb. salivarius INIA P183	Breast-fed infant faeces	Rodríguez et al., 2012

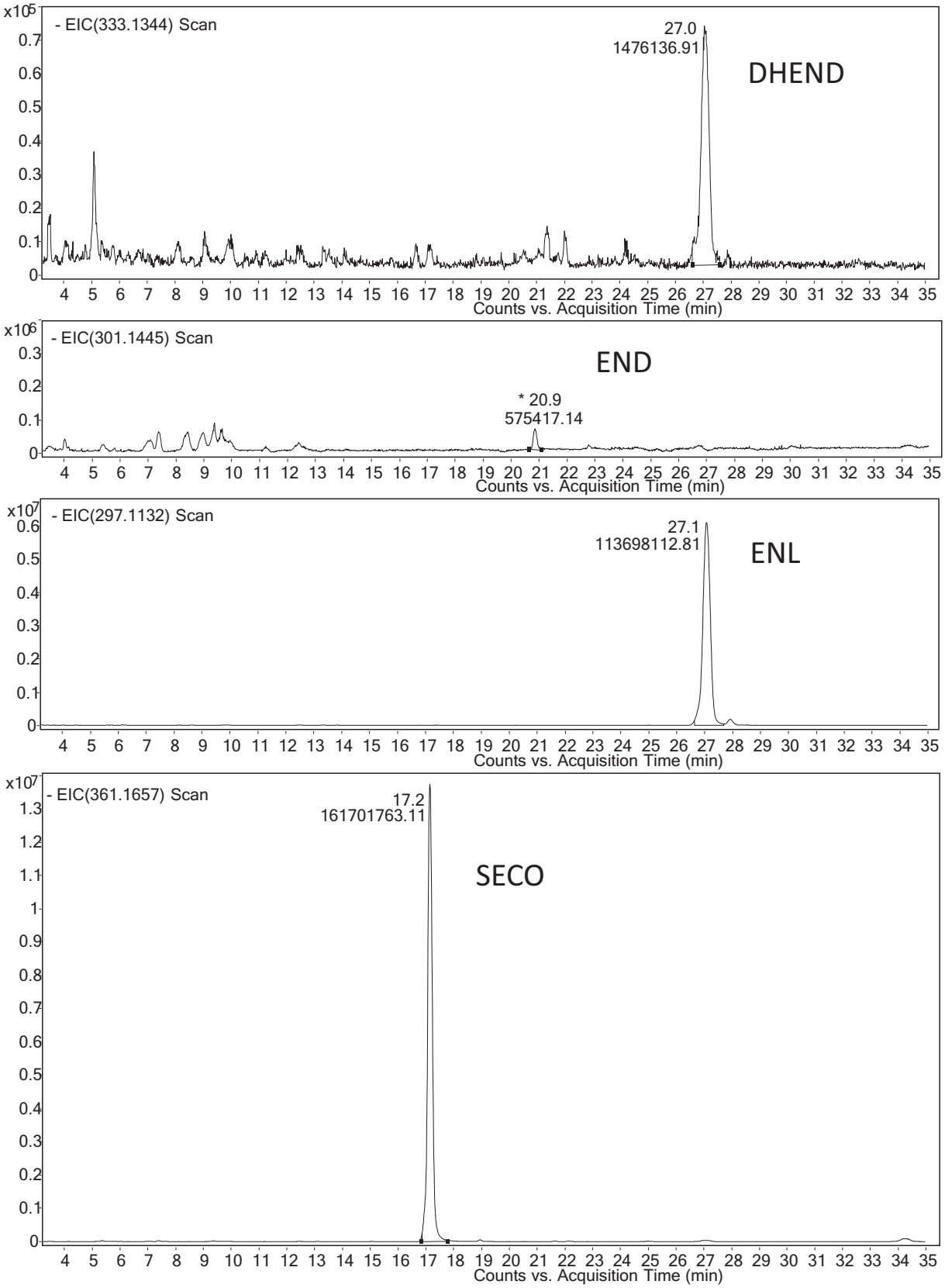

Fig. 1. Identification of secoisolariciresinol (SECO) and products of its metabolism by *Lb. salivarius* INIA P448. HPLC-ESI/MS chromatograms showing production of dihydroxyenterodiol (DHEND), enterodiol (END) and enterolactone (ENL), and remaining SECO.

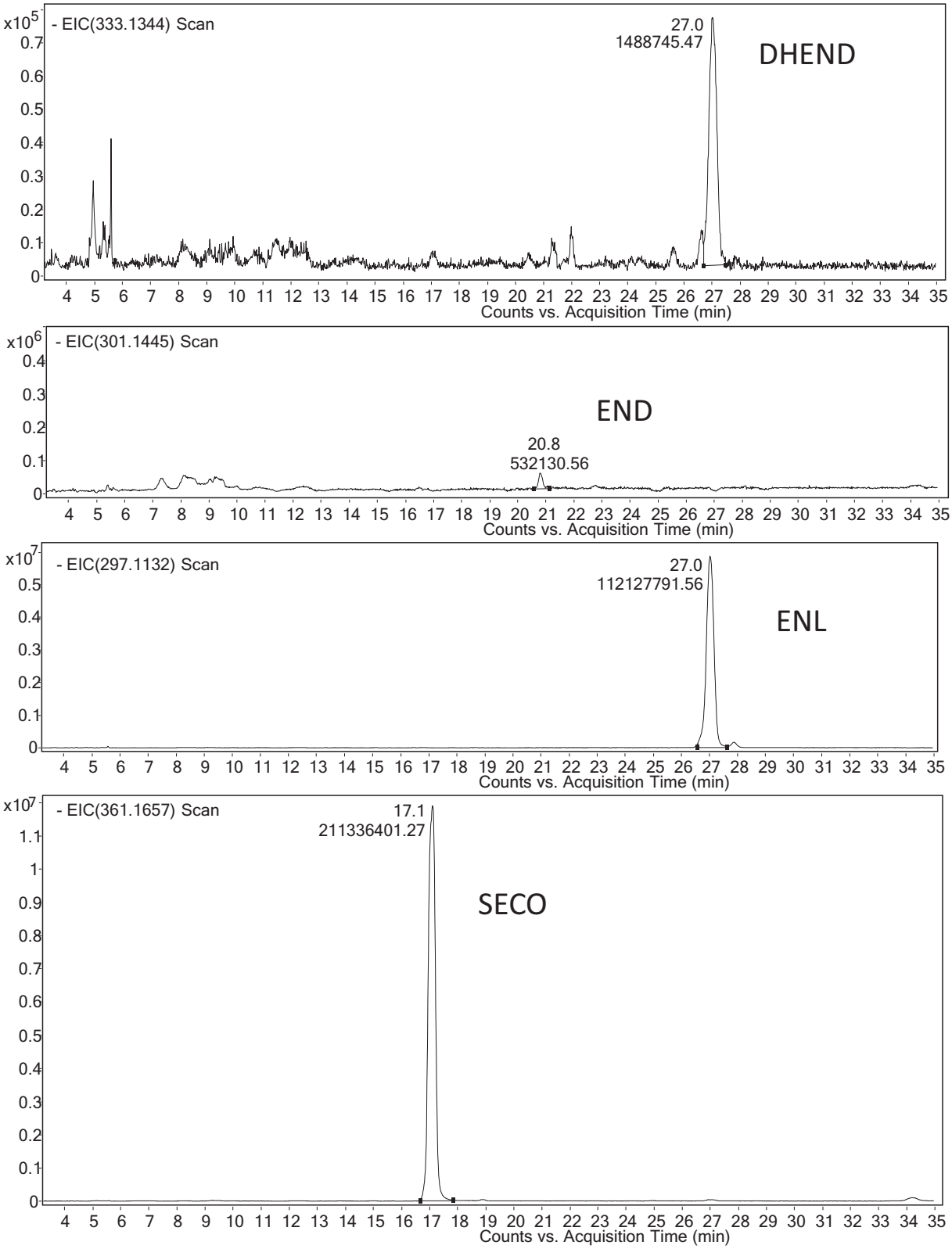

Fig. 2. Identification of secoisolariciresinol (SECO) and products of its metabolism by *B. catenulatum* INIA P732. HPLC-ESI/MS chromatograms showing production of dihydroxyenterodiol (DHEND), enterodiol (END) and enterolactone (ENL), and remaining SECO.

2.5. Identification and quantification of lignans by HPLC-ESI/MS analysis

Extraction of lignans was performed according to Gaya et al. (2016a). All samples were analysed by HPLC-ESI/MS. Separation of phenolic compounds was achieved on a reverse phase Nova-Pak C18 column (300 mm × 3.9 mm, 4 μm) (Waters, Barcelona, Spain) and the analytical conditions were based on those described by Gaya et al. (2016b). Mass spectra were obtained using a LC-MS Agilent 1200 (Palo Alto, CA, USA) chromatography system equipped with a quaternary pump (G1311A), a degasser (G1322A), a thermostated autosampler

Table 2
Characteristics of lignans and enterolignans detected in the present work.

Compound[a]	Formula	HPLC-ESI/MS	
		$[M-H]^-$ (*m/z*)	Retention time (min)
SDG	$C_{32}H_{46}0_{16}$	685.3	9.7
SECO	$C_{20}H_{26}0_6$	361.2	17.2
MAT	$C_{20}H_{22}0_6$	357.1	25.0
DHEND	$C_{18}H_{22}0_6$	333.1	26.6
END	$C_{18}H_{22}0_4$	301.1	20.8
ENL	$C_{18}H_{18}0_4$	297.1	27.1

[a] Secoisolariciresinol diglucoside (SDG), secoisolariciresinol (SECO), matairesinol (MAT), dihydroxyenterodiol (DHEND), enterodiol (END), enterolactone (ENL).

Table 3
Metabolism of lignans from flax extracts (LinumLife EXTRA) after incubation with a collection of 20 strains of *Bifidobacterium*. Concentrations determined by means of HPLC ESI/MS and expressed as mean ± standard deviation.

Bifidobacterium strains	SECO (μM)[a]	END (μM)[b]
B. adolescentis INIA P549	70.5 ± 7.8	n.d.
B. animalis INIA P490	663.0 ± 38.2	n.d.
B. animalis INIA P913	929.0 ± 302.6*	n.d.
B. bifidum INIA P466	64.5 ± 9.2	8.0 ± 2.8
B. breve INIA P12	688.0 ± 66.5	n.d.
B. breve INIA P367	1287.0 ± 128.7*	n.d.
B. breve INIA P763	66.5 ± 20.5	n.d.
B. breve INIA P776	1235.0 ± 346.5*	n.d.
B. catenulatum INIA P732	918.0 ± 217.8*	2.0 ± 1.4
B. catenulatum INIA P765	54.5 ± 30.4	n.d.
B. catenulatum INIA P825	438.0 ± 19.8	n.d.
B. dentium INIA P883	144.0 ± 50.9	n.d.
B. infantis INIA P593	91.5 ± 2.1	n.d.
B. infantis INIA P722	84.0 ± 4.2	n.d.
B. infantis INIA P728	129.0 ± 25.5	n.d.
B. longum INIA P9	113.0 ± 15.6	n.d.
B. longum INIA P750	69.0 ± 33.9	n.d.
B. pseudocatenulatum INIA P846	593.0 ± 123.0	n.d.
B. pseudocatenulatum INIA P946	6346.0 ± 3621.8*	n.d.
B. pseudolongum INIA P2	309.0 ± 123.0	11.0 ± 1.4
Control without bifidobacteria	92.0 ± 7.1	n.d.

[a] Secoisolariciresinol (SECO).
[b] Enterodiol (END), n.d. no detected.
* Significantly different from control (Dunnett test, $p < 0.01$).

Table 4
Concentrations of lignan metabolism compounds after incubation with SECO (5 mM).

Strains	Compound (μM)[a]			
	SECO	DHEND	END	ENL
B. bifidum INIA P466	3325 ± 632	25 ± 10	8 ± 1	1734 ± 174
B. catenulatum INIA P732	2089 ± 467	41 ± 12	21 ± 2	2321 ± 98
B. pseudolongum INIA P2	3829 ± 541	18 ± 2	7 ± 1	973 ± 71
Lb. gasseri INIA P508	2314 ± 245	17 ± 9	6 ± 1	147 ± 49
Lb. salivarius INIA P448	2943 ± 651	24 ± 6	8 ± 1	1813 ± 111
Lb. salivarius INIA P183	3521 ± 214	19 ± 2	10 ± 1	1242 ± 87
B. breve INIA P12	4873 ± 132	n.d.	n.d.	n.d.
B. infantis INIA P728	4965 ± 41	n.d.	n.d.	n.d.

[a] Concentrations determined by means of HPLC ESI/MS and expressed as mean ± standard deviation, secoisolariciresinol (SECO), dihydroxyenterodiol (DHEND), enterodiol (END), enterolactone (ENL).

(G1367B), a thermostated column compartment (G1316A), a photodiode array detector (G1315B), and a quadrupole mass spectrometer (QTOF Agilent G6530A) with an electrospray ionization (ESI) interface and Masshunter Data Acquisition and Qualitative Analysis (B.40.0) as control software. Other ESI/MS parameters were as follows: range acquisition 100–1000 *m/z*, gas temperature 350 °C, gas flow 10 L/min, nebulizer 45 psig, sheath gas temperature 350 °C, sheath gas flow 11 L/min, capillary voltage 3500 V, and fragmentation voltage 120 V. The mass spectrometer operated in the negative ion mode.

Quantification of SECO, MAT, END and ENL was made using the external standard calibration curves, with commercial standards. DHEND was quantified using the calibration curves of the most similar compound, i.e. END.

2.6. Statistical analysis

Statistical analysis was performed using of SPSS Statistics 22.0 software (IBM Corp., Armonk, NY, USA). Data were analysed by ANOVA using a general linear model (GLM). Comparison of means towards the control was carried out by Dunnett's test for a confidence interval of 99%.

3. Results

3.1. Screening of enterolignan production from flax extract by Bifidobacterium strains

We analysed the metabolism of lignans by 20 bifidobacterial strains belonging to 10 different species, selected from INIA culture collection for their biotechnological and probiotic properties. The screening for lignan metabolism was carried out on a lignan extract from flax seeds, containing as main lignans SDG and SECO, smaller amounts of pinoresinol and matairesinol, and no traces of DHEND, END and ENL (Gaya et al., 2017a). An increase of SECO with respect to control was observed in 14 out of the 20 strains tested, although the increase was significant just in five (Table 3), with the highest formation of SECO corresponding to *Bifidobacterium pseudocatenulatum* INIA P946. Three *Bifidobacterium* strains of three different species produced END from flax extract: *Bifidobacterium bifidum* INIA P466, *Bifidobacterium catenulatum* INIA P732 and *Bifidobacterium pseudolongum* INIA P2. Interestingly, only one of the bifidobacteria producing END was among those showing a significant increment in SECO. Production of ENL and DHEND was not observed in this screening in any of the bifidobacteria tested.

3.2. Enterolignan production from pure compounds by Lactobacillus and Bifidobacterium strains

The *Bifidobacterium* and *Lactobacillus* strains able to produce enterolignans from flax extract were subjected to further analysis using the pure lignans SECO and MAT as substrates.

The six strains that had previously showed enterolignan production from flax extract, showed production of END and ENL from SECO and the formation of the intermediate compound DHEND (Table 4, Figs. 1 and 2). On the contrary, the production of ENL from MAT and the transformation of SECO into MAT described elsewhere (Heinonen et al., 2001) were not observed in any of the tested strains. The two bifidobacteria non-producing END from lignan extract and the control without bacteria did not show production of enterolignans or DHEND from any of the compounds tested.

Of the metabolised SECO, the six strains were able to transform > 96% into ENL, resulting in lower concentrations of DHEND and END (Table 2). The concentration of DHEND was approximately 3-fold higher than that of END for all the enterolignan-producing strains. No strain consumed the whole of SECO, being *B. catenulatum* INIA P732 and *Lb. gasseri* INIA P508 the strains that consumed the greatest percentage of SECO (58.28% and 53.22% respectively) (Table 4).

In concordance with the greatest amount of SECO metabolised, the strain that yielded the highest concentration of ENL was *B. catenulatum* INIA P732, followed by *Lb. gasseri* INIA P508. The reduction of SECO could be correlated with the increase of ENL in the producing strains.

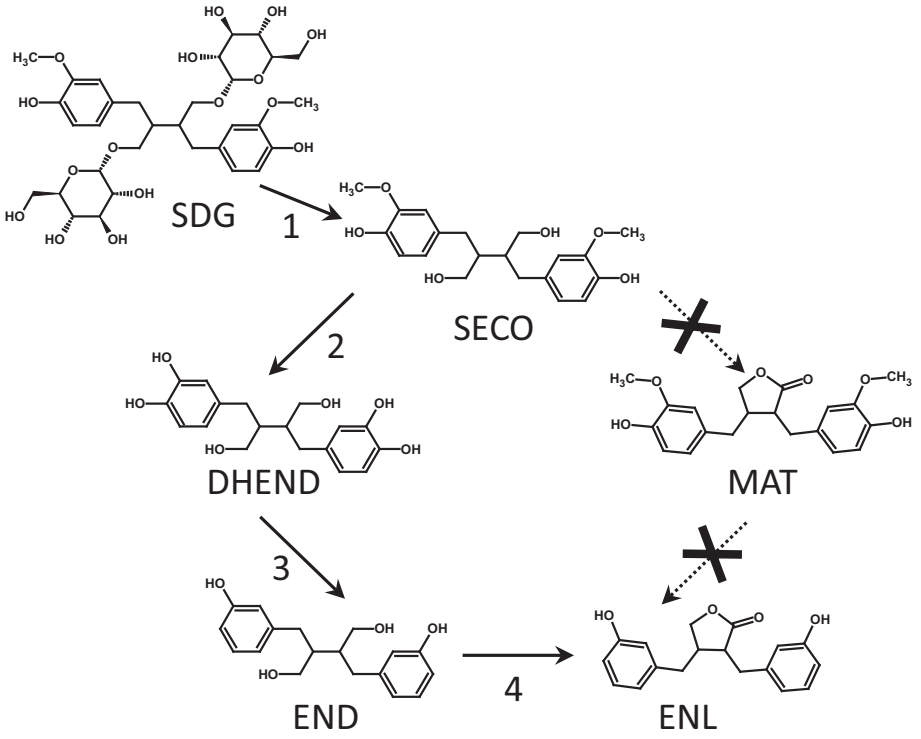

Fig. 3. Enterolignan metabolism observed in *Bifidobacterium* and *Lactobacillus* strains. Secoisolariciresinol diglucoside (SDG) is transformed into secoisolariciresinol (SECO) by means of deglycosylation reaction (1). Dihydroxyenterodiol (DHEND), enterodiol (END) and enterolactone (ENL) are produced afterwards by means of demethylation (2), dehydroxylation (3) and dehydrogenation (4) reactions. Neither production of matairesinol (MAT) from SECO nor production of ENL from MAT was detected.

4. Discussion

Enterolignans have been linked to the prevention of cardiovascular disease and colon, prostate and breast cancer (Aarestrup et al., 2013; Piller et al., 2006; Seibold et al., 2014; Vanharanta et al., 1999; Yoder et al., 2015). Enterolignans are produce by the intestinal microbiota from dietary lignans, that are common in Western diet, but there is an important interindividual variability in their production, which could be linked to differences in the microbiota composition. Therefore, the identification and use of bacteria for the biotransformation of lignan extracts into END and ENL is of high interest.

Recently we described the obtaining of END from flax extracts by the action of *B. adolescentis* INIA P784 (Gaya et al., 2017a), and three *Lactobacillus* strains able to produce END and ENL also from flax extracts (Bravo et al., 2017). In the present work, we extended the study including more bifidobacteria and exploring the metabolism with pure lignans.

Deglycosylation of the SDG present in flax extracts into SECO is the first step towards the formation of enterolignans. Several of the bifidobacteria tested showed the ability to increase the content of SECO of the flax extracts, accordingly to the frequency of this trait found in bifidobacteria (Gaya et al., 2017a). Nevertheless, the ulterior metabolism towards the formation of enterolignans was only observed on three of the tested bifidobacteria, with the detection of low amounts of END after incubation with flax extract. Both the ability to increase SECO concentration and the production of END by bifidobacteria seem to be a strain-specific trait. As opposed to the previous findings on *Lb. salivarius* and *Lb. gasseri* (Bravo et al., 2017), none of the bifidobacteria were able to produce ENL from flax extracts, and they produced END in lower amounts than those *Lactobacillus* strains. Interestingly, the ulterior incubation of the selected strains with pure SECO revealed the production of high levels of ENL, as well as the detection of DHEND and END, in both the *Lactobacillus* and the *Bifidobacterium* strains. The different behavior of bifidobacteria and lactobacilli when incubated with flax extract could be explained by an impairment of the growth of the bifidobacteria when the extract was present but not in presence of pure SECO, this could explain the lower production of END and the lack of production of ENL from flax extract. When using pure SECO as substrate, the most of the SECO fraction used by the strains was transformed into ENL, in agreement with previous results with the intestinal microbiota of adults and infants (Gaya et al., 2016b; Gaya et al., 2017b), which produced much more ENL than END or even only produced ENL.

The other pure lignan tested, MAT, has been described as a possible intermediate in the route of transformation of SDG and SECO by intestinal bacteria (Quartieri et al., 2016), which can dehydroxylate MAT to obtain ENL. However, we did not observe ENL production from MAT by any of the *Lactobacillus* or *Bifidobacterium* strains producing enterolignans from SECO. The levels of MAT in the media remained the same after incubation with the tested strains, and it was not detected as an intermediate when SECO was used as precursor. Thus, it could be inferred that MAT does not form part of the enterolignan metabolic pathway of these strains.

The use of pure compounds such as SECO and MAT and the detection of DHEND and END in addition to ENL concur with the metabolic pathway already proposed by other authors (Clavel et al., 2006b). SDG is transformed into SECO by means of deglycosylation reaction and DHEND, END and ENL are produced afterwards by means of demethylation, dehydroxylation and dehydrogenation reactions (Fig. 3).

Both the three *Bifidobacterium* and the three *Lactobacillus* strains producing enterolignans from flax extract increased the production of these compounds, especially of ENL, when the substrate was pure SECO instead of flax extract. This suggests that a higher availability of SECO facilitate the production of ENL. Although an increase on the deglycosylation of pure SDG into SECO has been described not to influence the enterolignan production by intestinal microbiota (Quartieri et al., 2016), other studies have showed a relationship between higher SDG transformation from lignan extracts and enterolignan production by intestinal microbiota (Gaya et al., 2016b; Gaya et al., 2017b). Differences between pure SDG used by Quartieri et al. (2016) and flax extracts rich in SDG (Gaya et al., 2016b; Gaya et al., 2017b) must be considered, incubation of strains with pure SDG could facilitate its subsequent metabolism.

Metabolism from pure SECO revealed that certain *Lactobacillus* and

Bifidobacterium strains have an active lignan metabolism, far superior to that found with flax extracts. The greater availability of pure SECO facilitated their subsequent metabolism. However, the production of END and ENL from pure SECO is not viable due to the high cost. The combination of strains capable of producing high concentrations of SECO from SDG, such *B. pseudocatenulatum* INIA P946, together with any of the *Bifidobacterium* and *Lactobacillus* strains capable of transforming SECO into ENL would be of great interest in the development of functional foods containing enterolignans.

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Conflicts of interest

The authors declare no conflict of interest.

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