Differential Catabolism of an Anthocyanin-Rich Elderberry Extract by Three Gut Microbiota Bacterial Species

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ABSTRACT

Elderberries are good sources of anthocyanins, which are poorly absorbed in the upper gastrointestinal tract but extensively transformed into phenolic metabolites at colonic level. Since different gut microbiota strains have different metabolism, the catabolism of anthocyanins may lead to inter-individual differences in metabolite production. In this work, an anthocyanin-rich elderberry extract was incubated with three single gut microbial strains (*Enterobacter cancerogenous*, *Bifidobacterium dentium* and *Dorea longicatena*) up to four days, to assess differences in their phenolic metabolism. All the strains degraded the elderberry anthocyanins, but the metabolic pathways followed were different. Although some metabolites were common for all the strains, a wide disparity was observed in the kind and in the amount of several phenolic metabolites produced by each species. These *in vitro* preliminary results may be of help in the interpretation of the bioavailability of anthocyanins and give a clue to understand inter-individual variability in metabolite production.

KEYWORDS (3 to 5)

Anthocyanin; phenolic acids; gut microbiota; metabolism; inter-individual variability.

INTRODUCTION

Anthocyanins are a group of phenolic compounds responsible for the red-orange to blue-violet color shades of fruits and vegetables.[1] Cyanidin, peonidin, delphinidin, malvidin, petunidin, and pelargonidin glycosides are the most common anthocyanins distributed in plants.[2,3] Significant sources of anthocyanins are berries (*i.e.* blueberries, mulberries, blackberries, elderberry, strawberries), red grapes, wine, and pomegranate. [4] Epidemiological and intervention studies have confirmed that the consumption of anthocyanin-rich foods is associated with a decreased risk of cardiovascular diseases,[5–7] an improvement of the antioxidant status,[8,9] and an amelioration of cognitive performance in impaired individuals.[10,11] Despite their biological effects, pharmacokinetic studies have demonstrated poor absorption in the upper gastrointestinal tract[12] and a very low bioavailability of anthocyanins, ranging from 0.5% to 1% of the ingested dose.[13,14] Particularly, it has been showed that they undergo an extensive decomposition in the colonic tract by gut microbiota.[15] Intriguingly, it has been suggested that different strains as well as different substituents on the A-, B-, C-rings of the flavylium cation might have a role in driving different breakdown pathways of the anthocyanin structure.[16] The first pioneering work concerning anthocyanin incubation with human fecal inocula indicated very low amounts of the single aglycons after 24-h fermentation.[17] Conversely, despite different compounds have been hypothesized to derive from A-ring breakdown, namely phloroglucinol and its aldehyde, resorcinol,[15,17,18] and ferulic acid,[18] B-ring structure is more prone to the microbiota-mediated transformations. González-Barrio *et al.*[15] hypothesized that the microbial cyanidin B-ring-derived compounds are mainly phenolic acids, as mono- and di-hydroxybenzoic, mono- and di-hydroxyphenylpropionic, and mono- and di-hydroxyphenylacetic acids, among others.[13] Unfortunately, the use of fecal inocula does not allow clear elucidation of which and how the single gut bacteria strains can access and modify the structure of anthocyanins. Moreover, the metabolic fate of the anthocyanin-derived compounds cannot be drawn by the

rearrangements of different strains present into the inoculum. In the present work, three different anaerobic gut microbial strains, namely *Enterobacter cancerogenous* (EC), *Bifidobacterium dentium* (BD), and *Dorea longicatena* (DL), were individually incubated with an anthocyanin-rich elderberry extract with the goal of identifying and quantifying anthocyanins and their metabolic derivative compounds. This will shed light on the contribution of different bacteria strains to the formation of anthocyanin-derived metabolites.

MATERIAL AND METHODS (≤ 500 words)

Chemicals

All chemicals and solvents were of analytical grade. All solvents were purchased from Sigma-Aldrich (St. Louis, MO, USA). Cyanidin-3-glucoside, pyrogallol, 3,4-dihydroxybenzoic acid (protocatechuic acid), 3,4-dihydroxybenzaldehyde, 2-(4'-hydroxyphenyl)acetic acid, 3-hydroxybenzoic acid, 4-hydroxybenzaldehyde, 4-methoxybenzaldehyde, 3-(3'-hydroxyphenyl)propionic acid, and 3-hydroxycinnamic acid (*m*-coumaric acid) were purchased from Sigma-Aldrich. 2-(4'-Hydroxy-3'-methoxyphenyl)acetic acid (homovanillic acid) was purchased from Extrasynthese (Genay Cedex, France). Ultrapure water from MilliQsystem (Millipore, Bedford, MA, USA) was used throughout the experiment.

Cell Culture

Anaerobic cultures were prepared using filter-sterilized "MML" medium (Supplementary Table S1) amended with 2% elderberry extract (*Sambucus nigra* L., DDW WS35, Port Washington, WI, USA). Tubes were inoculated (0.5 mL inoculum into 10 mL medium) with freshly-grown cultures—prepared in the same medium supplemented with 10 mM glucose and 5-fold increased levels of tryptone, yeast extract and meat extract—of *Bifidobacterium dentium* ATCC 27534, *Dorea longicatena* DSMZ 13814 or *Enterobacter cancerogenous* ATCC 36316 (kindly supplied by J. Gordon,

Washington University, St. Louis, MO, USA), or left uninoculated as a control, then incubated at 37°C. Strains were chosen based on pilot experiments where these strains differentially degraded elderberry anthocyanins into 3,4-dihydroxybenzoic acid (protocatechuic acid, Supplementary Figure S1). In particular, DL and BD produced high and moderate amounts of protocatechuic acid, respectively, while EC degraded the amount of protocatechuic acid in the extract (Supplementary Figure S1). Samples (1.0 mL) were obtained using sterile 75% N_2 / 20% CO_2 / 5% H_2-flushed syringes, injected into 128 µL of 88% formic acid, chilled on ice, centrifuged to pellet cells, then 0.2 µm-filtered and stored at -80°C prior to analysis. Cultures derived from a single flask were assayed for each time point.

Sample preparation and UHPLC-MS/MS and –MS^n analyses

Cell media were extracted according to Sala et al.[19], but using acidified methanol (0.1% formic acid) instead of pure methanol. Samples were analyzed for quantification purposes by UHPLC DIONEX Ultimate 3000 equipped with a TSQ Vantage triple quadrupole mass spectrometer (Thermo Fisher Scientific Inc., San Jose, CA, USA), as fully reported in Brindani et al.[20] The tentative identification of some compounds was carried out using targeted full MS^2 experiments with the same instrument and analytical conditions. Quantification was performed with calibration curves of standard compounds, when available. When not available, the metabolites were quantified with the most similar compound in terms of molecular weight and structure.

Targeted full MS^3 experiments were carried out in order to confirm the identity of some anthocyanins. Experiments were carried out using an Accela UHPLC 1250 equipped with a linear ion trap-mass spectrometer (MS) (LTQ XL) fitted with a heated-electrospray ionization (ESI) probe (Thermo Fisher Scientific Inc.). Analytical details are provided at Supplementary Method S1.

Statistical analysis

The SPSS statistical package (SPSS Inc., Chicago, IL, USA, version 25) was used. Analyses were performed in triplicate. All data were expressed as mean ± SD. One-way ANOVA with Tukey HSD *post hoc* tests were carried out. Principal component analysis (PCA) with varimax was performed to explore the variability among bacterial strains at different time points.

RESULTS AND DISCUSSION

The set of compounds targeted and/or identified, and their spectrometric characteristics are presented in **Table 1**. Thirty compounds related to the anthocyanin profile of the elderberry extract (compounds **1, 2**, **3**, **4**, **5**, and **6**) and to the putative degradation pathway of elderberry anthocyanins (**7-20**, **22**, **24, 25-29**, **33**, **34** and **35**) were targeted by considering previous reports.[15,18,21,22] Eleven out of the 30 compounds were not found in the cultured samples (**11**, **12**, **14-17**, **24, 27**, **29**, **34**, and **35**), while up to 6 unexpected peaks were observed in the chromatograms (compounds **21**, **23**, **30-32**, and **36**). Peak areas for these non-targeted compounds changed in comparison with the uninoculated control medium and depending on the bacteria strains and time points, so they were considered to be potential isomers of the targeted metabolites derived from anthocyanin catabolism. However, although additional commercial standards were run and further *ad hoc* full MS^2 target experiments were carried out and compared with literature, only compounds **21** and **30** could be identified. Chromatographic and MS^2 spectra data for these unidentified compounds are reported in **Table 1**. Similarly, additional experiments were carried out in order to fully confirm the presence of peonidin glycosides (**5** and **6**) in the elderberry extract used. Although Olejnik et al.[23] reported the presence of these two compounds in an elderberry extract at trace levels, their presence in elderberry contrasted with the typical anthocyanin profile of this berry.[24-26] Therefore, in order to fully confirm the presence of peonidin derivatives in elderberry, a targeted MS^3 experiment was conducted. Chromatographic and MS^2 data for compounds **5** and **6** (Table 1) were

in agreement with the evidence reported by Olejnik et al.[23] The fragmentation pattern of both aglycones (MS^2 ion fragment at *m/z* 301) accounted for a sole MS^3 ion fragment at *m/z* 286 (Supplementary Figure S2), corresponding to the loss of the methyl moiety present on the B-ring of the peonidin aglycone. These data confirmed the presence of peonidin glycosides in elderberry extracts.

Table 1: Monitored compounds in cell culture samples.

ID	Compound	RT (min)	Parent Ion (m/z)	S-Lens	Quantifier Product Ion (m/z)	CE (V)	Qualifier Product Ion (m/z)	CE (V)	MS^2 Fragments	STD
			$[M]^+$							
1	Cyanidin-3,5-diglucoside	1.84	611	108	287	32				3
2	Cyanidin-3-sambubioside-5-glucoside	1.90	743	108	287	32				3
3	Cyanidin-3-glucoside	3.49	449	108	287	32				3
4	Cyanidin-3-sambubioside	3.56	581	108	287	32				3
5	Peonidin-3-glucoside	3.83	463	108	301	32				3
6	Peonidin-3-sambubioside	3.83	595	108	301	32				3
			$[M-H]^-$							
7	Catechol	0.90	109	68	108	20	81	20		8
8	Pyrogallol	0.99	125	68	124	24	97	15		8
9	3,4-Dihydroxybenzoic acid (Protocatechuic acid)	1.46	153	64	109	20	108	27		9
10	3,4-Dihydroxybenzaldehyde	2.06	137	80	108	30	92	30		10
11	2-(3',4'-Dihydroxyphenyl)acetic acid	2.22	167	60	123	17				N.F.
12	4-Hydroxybenzoic acid	2.41	137	70	93	16	91	5		N.F.
13	2-(4'-Hydroxyphenyl)acetic acid	2.58	151	51	107	12				13
14	2-(4'-Hydroxy-3'-methoxyphenyl)acetic acid (Homovanillic acid)	2.76	181	64	137	24	122	30		N.F.
15	4-Hydroxybenzyl alcohol	3.00	123	80	105	30	77	30		N.F.
16	3-Hydroxybenzoic acid	3.22	137	70	93	16	45	5		N.F.
17	4-Hydroxybenzaldehyde	3.22	121	80	92	30	108	30		N.F.
18	3,4-Dihydroxycinnamic acid (Caffeic acid)	3.46	179	62	135	19	134	28		30
19	4-Methoxybenzaldehyde	3.47	135	80	106	30	79	30		19
20	4-Hydroxy-3-methoxy benzoic acid (Vanillic acid)	3.55	167	60	123	17	152	18		14
21	2-(2'-Hydroxyphenyl)acetic acid	3.62	151	51	107	12			107, 123	13
22	2,4,6-Trihydroxybenzaldehyde (Phloroglucinaldehyde)	3.84	153	80	83	30	151	30		17

23	N.I.	3.85	151	51	107	12			107, 83	26
24	3-(3',4'-Dihydroxyphenyl)propanoic acid (Dihydrocaffeic acid)	3.95	181	64	137	14	119	13		N.F.
25	3-(3'-Hydroxyphenyl)propionic acid	4.22	165	48	119	20	121	15		26
26	4-Hydroxycinnamic acid (*p*-Coumaric acid)	4.22	163	62	119	19	93	38		30
27	4-Hydroxy-3,5-dimethoxybenzoic acid (Syringic acid)	4.30	197	70	182	20	153	26		N.F.
28	3-(4'-Hydroxy-3'-methoxyphenyl)propanoic acid (Dihydroferulic acid)	4.35	195	73	136	21	151	22		14
29	3-Hydroxycinnamic acid (*m*-Coumaric acid)	4.52	163		119		93			N.F.
30	3-Hydroxy-4-methoxy-benzoic acid (Isovanillic acid)	4.55	167	60	123	17	152	18	152, 123, 108	9
31	N.I.	4.71	151	51	107	12			107	26
32	N.I.	4.94	137	70	93	16	91	5	93	16
33	4-Hydroxy-3-methoxycinnamic acid (Ferulic acid)	4.96	193	71	134	19	178	18		30
34	3-Hydroxy-4-methoxycinnamic acid (Isoferulic acid)	5.00	193	71	134	19	178	18		N.F.
35	4-Hydroxy-3,5-dimethoxycinnamic acid (Sinapic acid)	5.30	223	94	179	31	208	25		N.F.
36	N.I.	5.94	181	64	137	24	122	30	137	14

Legend: N.I., not identified. These N.I. compounds were observed while analyzing other compounds with the same molecular and fragments ions; they were not identified when subjected to MS^2 analysis (MS^2 fragments) and in comparison to other commercially available isomers. They were quantified using the most similar standard according to their molecular weight and their putative structure. N.F., Not Found; STD means the available standard compound used for quantification.

Quantitative data indicated that cyanidin glycosides accounted for the majority (99%) of the anthocyanins in the elderberry extract (**Table 2**). Considering the 0-day time point, elderberry extract compounds **3** and **4** presented concentrations about 6- and 18-fold higher than compounds **2** and **1**. These data are in agreement with previous studies characterizing the anthocyanin profile of elderberry fruits.[25,26] Peonidin derivatives accounted for 1% of the anthocyanin profile, in line with previous data.[23] Univariate analyses evidenced several statistically significant differences in the concentration of the 25 compounds detected both among the control medium and the three gut microbiota strains, and among the different collection time points (**Table 2**).

Table 2: (Poly)phenol concentration after incubations of cultures or uninoculated control medium. Different lowercase letters indicate statistically significant differences ($p < 0.05$) among treatments, at the same incubation time, for each compound; while different uppercase letters account for differences within treatment at different time points.

ID	CONTROL (µmol/L)			*Enterobacter cancerogenous* (µmol/L)			*Bifidobacterium dentium* (µmol/L)			*Dorea longicatena* (µmol/L)		
	0 day	1 day	4 day	0 day	1 day	4 day	0 day	1 day	4 day	0 day	1 day	4 day
1	14.8±0.9 aA	9.7±0.9 aB	7.7±0.7 aB	13.6±1.7 aA	10.9±1.2 aAB	6.7±0.0 bB	9.9±0.6 bA	1.3±0.1 bB	0.0±0.0 dC	14.8±0.1 aA	10.4±0.8 aB	4.6±0.5 cC
2	46.6±2.6 aA	31.7±3.6 aB	29.3±1.8 aB	42.6±5.0 aA	35.0±3.2 aA	27.6±1.9 aB	31.0±0.1 bA	4.6±0.5 bB	0±0 bC	46.1±3.6 aA	34.0±2.5 aB	23.3±2.3 aC
3	272.0±4.5 abA	226.4±9.6 aB	185.5±11.4 aC	268.5±23.1 abA	181.6±14.7 bB	67.8±2.1 bC	234.6±7.5 bA	36.6±4.1 cB	0.3±0.04 dC	277.2±2.1 aA	206.2±19.7 abB	47.7±4.5 cC
4	242.3±5.2 aA	216.6±6.7 aB	199.8±10.5 aB	231.2±33.5 aA	159.9±18.2 bA	64.3±0.4 cB	215.8±6.4 aA	207.4±23.8 abA	99.8±14.2 bB	246.5±2.8 aA	202.2±15.6 abB	71.6±8.7 bcC
5	1.0±0.1 aA	0.6±0.0 aB	0.5±0.1 aB	1.0±0.1 aA	0.5±0.1 aB	0.2±0.0 bB	0.7±0.1 aA	0.1±0.0 bB	0.0±0.0 cB	1.0±0.0 aA	0.6±0.1 aB	0.2±0.0 bC
6	0.3±0.0 aA	0.2±0.0 aB	0.2±0.0 aB	0.3±0.0 aA	0.2±0.0 aAB	0.2±0.0 aA	0.2±0.0 aB	0.3±0.0 aA	0.1±0.0 bC	0.3±0.0 aA	0.2±0.0 aB	0.1±0.0 bC
7	9.2±0.3 aAB	8.9±0.7 abB	10.3±0.3 bA	8.0±0.8aA	6.9±0.2 cA	0.0±0.0 dB	8.0±0.0 aB	9.5±1.0 aA	11.4±0.8 aA	8.5±0.1 aA	7.5±0.6 bcA	7.9±0.5 cA
8	0.0±0.0	0.0±0.0 b	0.0±0.0 b	0.0±0.0 cC	1.1±0.0 aB	1.8±0.1 aA	0.0±0.0	0.0±0.0 b	0.0±0.0 b	0.0±0.0	0.0±0.0 b	0.0±0.0 b
9	11.5±0.9 aB	11.2±0.9 cB	15.7±1.5 bA	11.3±1.6 aA	6.6±0.9 dB	4.4±0.4 cB	11.0±0.4 aB	25.1±3.2 bA	21.2±3.2 bA	11.9±0.5 aB	39.7±3.3 aA	42.7±3.3 aA
10	0.3±0.1 aB	0.4±0.1 cB	0.6±0.1 bA	0.4±0.1 aC	0.7±0.1 bB	1.0±0.0 aA	0.4±0.0 aB	0.9±0.1 abA	0.9±0.1 aA	0.4±0.0 aB	1.2±0.1 aA	1.3±0.2 aA
13	0.0±0.0	0.0±0.0 b	0.0±0.0b	0.0±0.0	0.0±0.0 b	0.0±0.0 b	0.0±0.0 aA	7.5±1.0 aB	8.1±0.02 aA	0.0±0.0	0.0±0.0 b	0.0±0.0 b
18	42.7±3.4 aB	47.3±3.1 cB	69.9±7.6 cA	45.7±5.2 aA	18.7±1.9 dB	7.9±0.9 dB	39.8±2.9 aC	84.7±7.4 bB	111.6±4.0 bA	46.9±0.5 aB	2153.5±301.8 aA	2240.2±309.9 aA
19	0.0±0.0	0.0±0.0 b	0.0±0.0 b	0.0±0.0	0.0±0.0 b	0.0±0.0 b	0.0±0.0 B	0.0±0.0 bB	0.1±0.01 bA	0.0±0.0 B	1.6±0.2 aA	1.9±0.2 aA
20	276.7±31.3 aA	190.2±24.5 aB	210.9±22.7 aAB	273.7±7.8 aA	0.0±0.0 bB	0.0±0.0 bB	0.0±0.0 b	0.0±0.0 b	0.0±0.0 b	232.8±33.3 aA	0.0±0.0 bB	0.0±0.0 bB
21	4.9±0.2 aAB	3.9±0.6 aB	6.2±0.6 bA	4.7±0.6 aA	4.5±0.6 aA	5.9±0.8 bA	3.7±0.5 aB	3.6±0.5 aB	8.0±0.6 aA	4.4±0.4 aA	3.7±0.3 aA	4.8±0.7 bA
22	0.9±0.1 bC	3.4±0.5 cB	8.9±0.6 aA	0.8±0.1 bC	2.4±0.2 dB	4.7±0.5 bA	3.9±0.3 aC	19.9±2.2 aA	9.3±0.7 aB	0.85±0.1 bC	6.7±0.7 bB	11.9±1.6 aA
23	0.0±0.0 cC	2.5±0.3 cB	5.8±0.7 aA	0.0±0.0 cA	1.9±0.2 cB	3.1±0.3 bC	2.6±0.2 aC	12.3±1.3 aA	5.9±0.6 aB	1.3±0.2 bC	4.3±0.3 bB	6.8±0.7 aA
25	1.6±0.2 bA	1.2±0.2 dA	1.2±0.1 dA	1.4±0.1 bB	2.6±0.4 bA	3.3±0.1 bA	3.1±0.2 aC	6.1±0.7 aB	44.5±3.4 aA	1.9±0.2 bA	1.8±0.1 cA	2.0±0.2 cA
26	2.4±0.2 abAB	2.2±0.2 bB	2.9±0.3 bA	1.6±0.3 bA	0.9±0.04 cB	1.1±0.1 cAB	2.8±0.2 aC	4.3±0.4 aB	6.1±0.5 aA	2.2±0.3 abB	4.02±0.5 aA	4.9±0.4 aA
28	53.6±6.55 bcB	97.8±13.2 cA	82.9±8.1 cA	43.0±6.7 cC	106.3±14.2 cB	223.1±23.7 bA	266.9±19.8 aC	1424.9±134.3 aA	720.1±69.7 aB	62.1±5.8 bC	315.4±35.1 bB	742.0±70.0 aA
30	0.2±0.0 aB	0.3±0.0 aD	0.2±0.0 aD	0.2±0.0 cA	4.4±0.4 bA	9.8±0.3 aA	4.0±0.2 bB	27.8±4.7 aC	34.9±2.8 aC	0.2±0.0 cB	6.3±0.6 bB	17.6±1.6 aB
31	0.0±0.0	0.0±0.0 b	0.0±0.0 b	0.0±0.0 C	11.0±0.4 aB	6.4±0.6 aA	0.0±0.0	0.0±0.0 b	0.0±0.0 b	0.0±0.0	0.0±0.0 b	0.0±0.0 b

32	206.7±21.5 aAB	164.7±7.7 cB	218.5±23.8 bA	196.2±26.2 aA	226.9±32.9 bA	221.2±11.0 bA	168.9±6.8 aB	341.5±36.6 aA	457.6±41.5 aA	193.7±12.7 aA	166.7±21.4 bcA	164.6±19.3 bA
33	1.7±0.2 aA	1.2±0.1 aB	1.1±0.1 aB	1.6±0.2 aA	1.2±0.1 aB	1.2±0.1 aB	1.2±0.1 aA	1.4±0.2 aA	1.2±0.1 aA	1.7±0.1 aA	1.3±0.1 aB	1.0±0.1 aB
36	0.0±0.0	0.0±0.0 b	0.0±0.0 b	0.0±0.0	0.0±0.0 b	0.0±0.0 b	0.0±0.0	0.0±0.0 b	0.0±0.0 b	0.0±0.0 B	140.2±15.2 aA	117.2±9.5 aA

Data are expressed as mean ± SD of three independent measurements.

Notably, lower anthocyanin concentrations (for compounds **1**, **2**, **3**, and **5**) were measured in BD cultures compared to control medium, EC, and DL cultures at the 1- and 4-day collection points, as well as for compound **4** in comparison to EC and the control medium at 4-day collection point. Among the phenolic acids changing during the incubation period, protocatechuic (**9**) and caffeic (**18**) acids were significantly higher in DL compared to BD, control medium, and EC cultures after 4-days incubation. Unknown compound **32** showed higher concentrations at 1-day and 4-day after BD incubation with respect to EC, control, and DL incubations. Last, some compounds like 3-(3'-hydroxyphenyl)propionic acid (**25**), *p*-coumaric acid (**26**) and ferulic acid (**33**) evidenced slight differences among time points for some treatments as well as among treatments for some collection points (**Table 2**).

A multivariate PCA was carried out to better understand the variability in the metabolic pathway of anthocyanin degradation for each gut microbial strain (**Figure 1**). Three principal components (PCs) explained up to 83.5% of the total variability. PC1 accounted for 39.9% of the observed variability and was positively loaded from some phenolics (**13**, **22**, **23**, **25**, **26**, **32**, and **28**), while negatively loaded from most of the anthocyanins (**1**, **2**, **3**, and **5**) (**Figure 1A, 1B**). PC2 explained 23.4% of the total variability and had positive loadings from protocatechuic acid (**9**), caffeic acid (**18**), methoxybenzaldehyde (**19**), and compound **36** (**Figure 1A**). PC3 accounted for 20.2% of the observed variability and was mostly loaded positively from catechol (**7**) and the anthocyanin sambubioside (**4** and **6**), whereas negatively from pyrogallol (**8**) and compound **31** (**Figure 1B**). Individual scores for each gut bacteria strain and time point revealed interesting insights with regard to the putative degradation pathways of anthocyanins, as compared to the control media (**Figures 1C** and **1D**). BD-mediated anthocyanin degradation was related to PC1 loads and, thus, to an extensive catabolism of anthocyanins and to increasing amounts of monohydroxylated phenolics (**13**, **25**, and **26**) and C6-C3 backbones (**25**, **26**, and **28**) along the 4-day incubation. DL metabolism,

associated with positive scores for PC2, entailed a mild degradation of the anthocyanin fraction but a high production of dihydroxylated phenolic acids (**9** and **18**) and C6-C1 scaffolds (**9** and **19**). EC catabolism, linked mainly to PC3 scores, accounted for an increasing production of pyrogallol (**8**) and an intense metabolism of catechol (**7**), as opposed to the rest of the bacterial strains, which did not evidence degradation pathways related to these two phenols.

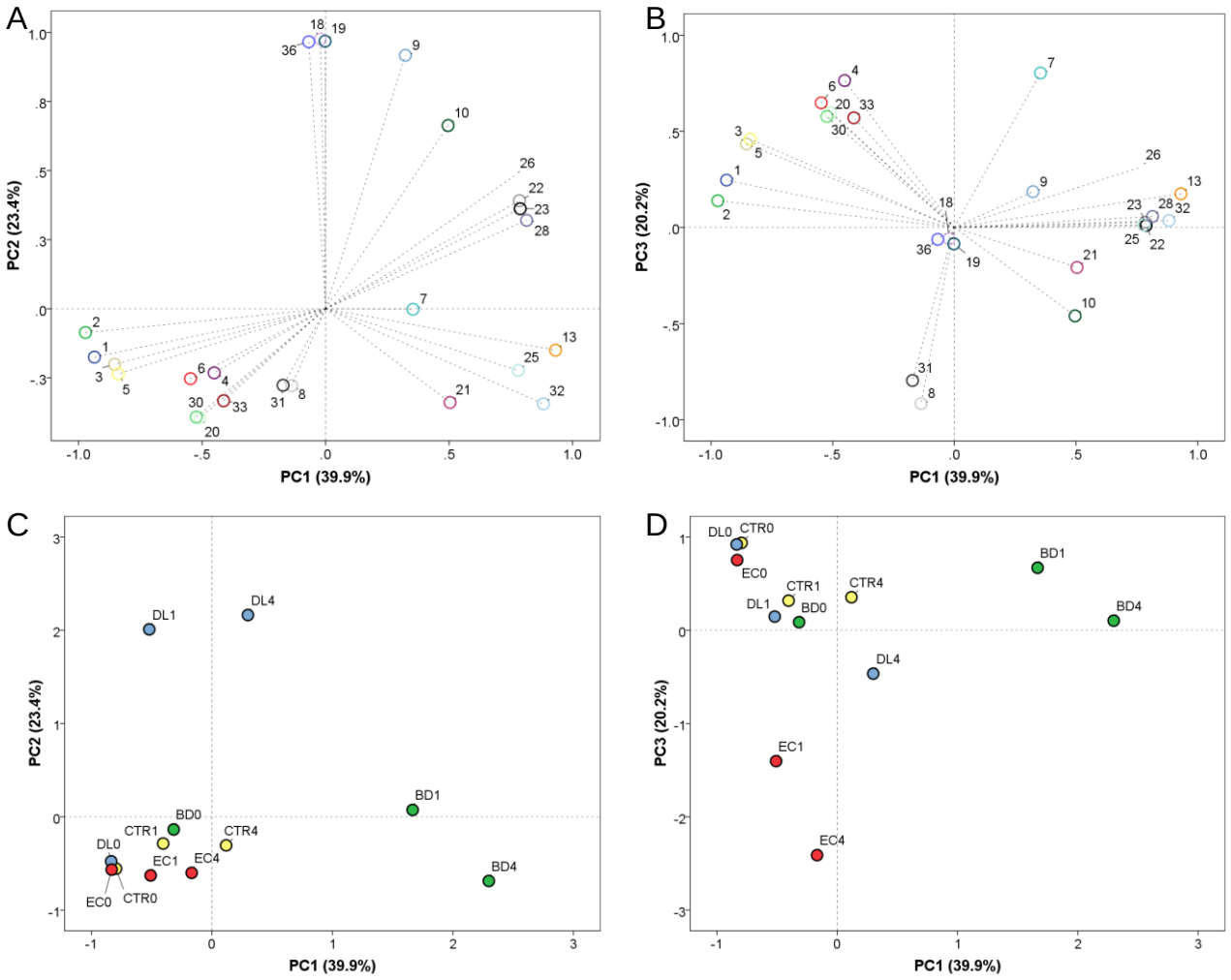

Figure 1. Loading plots (A, C) and score plots (B, D) of the first three PCs obtained from the PCA with varimax of the considered phenolic metabolites and bacterial strains activity. Legend: CTR, control cell medium; BD, *Bifidobacterium dentium*; DL *Dorea longicatena*; EC, *Enterobacter cancerogenous*; PC, principal component. Numbers close to control medium and bacteria acronyms (0, 1 and 4) indicate the days of the medium samples collection after incubation with anthocyanins. The legend relative to the phenolic metabolite numbers is available in **Table 1**.

The microbial degradation of anthocyanins may follow several different pathways, as reported in previous *in vivo* and *in vitro* studies,[15,18,22] and this aspect highly influences anthocyanin bioavailability. Following the pattern described by González-Barrio *et al.* for raspberry anthocyanins incubated with human fecal suspensions,[15] our results suggested that only EC was able to degrade cyanidin derivatives to yield pyrogallol (**8**) but not catechol (**7**), which was completely degraded. Cyanidin can also be degraded by releasing protocatechuic acid (**9**),[15] which increased along the incubation time in BD and DL, while it decreased in EC. In this sense, De Ferrars et al.[18] reported a decreasing trend for protocatechuic acid amounts in fecal samples collected for 2 days after supplementation of human volunteers with $^{13}C_5$-labelled cyanidin-3-glucoside. Regarding caffeic acid (**18**), a key intermediate of anthocyanin degradation,[22] it showed a very significant increment when elderberry extract was incubated with DL, with a moderate increase registered also for BD. Dihydroferulic acid (**28**) also increased notably in presence of the three tested inocula, while ferulic acid (**33**) was not a relevant metabolite. De Ferrars et al.[18] evidenced similar results for caffeic acid in fecal samples of volunteers supplemented with $^{13}C_5$-labelled cyanidin-3-glucoside, but they recovered very high amounts of ferulic acid and not of dihydroferulic acid, although the dehydrogenation of the double bond has been previously hypothesized in the potential microbial metabolic pathways of cyanidin-based anthocyanin.[14] The microbial degradation of cyanidin also led to the formation of phloroglucinaldehyde (**22**),[18] while in our study this compound increased for EC and DL along the 4-day incubation, remaining stable after BD incubation. Moreover, phloroglucinaldehyde has found to be metabolized to methoxybenzaldehyde (**19**),[18] a pathway that was confirmed mainly for DL and just scarcely for BD, while it was not found for EC. Concerning 3,4-dihydroxybenzaldehyde (**10**), an aldehyde putatively derived from protocatechuic acid, it increased for all the tested strains, as did 3-(3'-hydroxyphenyl)propionic acid (**25**).[18]

To better describe the metabolic transformations carried out by each bacterial strain, efforts were made to identify the unexpected metabolites. Compound **32** ($[M-H]^-$ at *m/z* 137) was hypothesized to be 2-hydroxybenzoic acid, as it might be formed from phloroglucinaldehyde following the pathway reported by De Ferrars et al.[18] and leading to 2-hydroxy-4-methoxybenzaldehyde formation. This same metabolic pathway may also be linked with the presence of 2-(2'-hydroxyphenyl)acetic acid (**21**). Similarly, compound **36** ($[M-H]^-$ at *m/z* 181), which was only present in DL samples, was hypothesized to be 2-(3'-hydroxy-4'-methoxyphenyl)acetic acid (aka homoisovanillic acid) since its retention time and fragment ions did not match with those of other isomers expected somehow from cyanidin degradation,[15,18,22] like 3-(3',5'-dihydroxyphenyl)propionic acid, 3-(2',4'-dihydroxyphenyl)propionic acid, and 3-(4'-hydroxyphenyl)lactic acid. Compound **36** might be related to the degradation pathways yielding other 3'-hydroxy-4'-methoxyphenyl structures (i.e., isovanillic acids).[15,18,22] Structure for compound **30** ($[M-H]^-$ at *m/z* 167) did not match with the analytical features of the isomers labelled as putative metabolites (i.e., 2-hydroxy-4-methoxyphenylbenzoic acid, 3-hydroxymandelic acid, 2-(2',4'-dihydroxyphenyl)acetic acid and 2-(3',5'-dihydroxyphenyl)acetic acid), but based on its MS^2 fragmentation pattern it has been identified as 3-hydroxy-4-methoxy-benzoic acid (isovanillic acid). On contrary, no one of the 2 parent ions $[M-H]^-$ at *m/z* 151 (**23** and **31**) could be assigned to an isomer of 4-hydroxyphenylacetic acid (**13**) or to a methoxybenzoic acid taking into account their retention times and fragmentation patterns.

The interplay between anthocyanin catabolism by the gut microbiota and the microbial modulation caused by anthocyanin consumption is well-known.[27] But, despite the different capability to metabolize anthocyanins of the three bacterial species assessed, there is no information linking them to differences in the phenolic metabolism of anthocyanin-rich sources. A previous report[28] demonstrated that *Clostridium saccharogumia* DSM 17460 and *Eubacterium ramulus* DSM 16296

have a different capability to degrade cyanidin-3-glucoside. The metabolic pathways related to these two bacterial strains were different from those observed when this compound was incubated *in vitro* with human fecal slurries.[28] Incubation of cyanidin-rutinoside with human gut microbiota led to a marked increase of *Bacteroides*, while a limited bifidogenic effect.[29] Nevertheless, high levels of Bifidobacteria have been associated with increased levels of anthocyanin-derived microbial catabolites upon consumption of red wine.[30] It has been recently hypothesized that differences in the metabolism of anthocyanins could be related to the gut microbiota composition of each individual and, in particular, to the relative abundance of *Bacteroides*.[29] However, to date, there is a lack of information on the inter-individual differences in the production of anthocyanin-derived metabolites by specific gut microbiota strains or by specific enterotypes. This work contributed hence to gain insights in the different pathways that may occur in gut microbiota-mediated anthocyanin metabolism.

A point worth mentioning is the confirmation of the presence of peonidin glycosides in elderberry extracts, in line with Olejnik et al.[23] The two peonidin derivatives identified have been previously identified in urine samples of volunteers consuming elderberry extracts.[21,31] Their presence in circulation upon consumption of elderberry has been related to the 3'-*O*-methylation of cyanidin derivatives, not affecting the glycosylation pattern of the anthocyanin.[21,31] However, this novel evidence on elderberry composition leaves the door open to hypothesize that not all the peonidin glycosides found in circulation are the result of cyanidin metabolism. Actually, the presence of peonidin glycosides in circulation upon consumption of elderberry extracts could be partially associated with the direct absorption of these compounds at gastrointestinal level, without further methylation. Further work is thus required to fully understand the metabolic reactions occurring at human level to elderberry anthocyanins and, in particular, to cyanidin glycosides.

In conclusion, the present study highlighted a considerable variability in the type and in the concentration of the anthocyanin degradation products among three gut bacterial strains. These findings support the idea that the presence of certain strains as well as the ecological competition among them are key factors at the basis of the inter-individual variability in anthocyanin bioavailability. Further studies are required in order to fully elucidate the degradation pathways of the most representative bacterial species and their relation to different gut microbiome compositions. Moreover, the effect of the food matrix or other sources of anthocyanins on the catabolism by individual bacterial strains of anthocyanins should be further explored.

ABBREVIATIONS USED

BD, *Bifidobacterium dentium*; DL, *Dorea longicatena*; EC, *Enterobacter cancerogenous*; PC, principal component; PCA, principal component analysis.

SUPPORTING INFORMATION DESCRIPTION

Supplementary Table S1. MML medium composition.

Supplementary Figure S1. Protocatechuic acid (PCA) production/degradation following elderberry anthocyanin metabolism by different gut microbiota strains or inoculated controls.

Supplementary Figure S2. Chromatograms and MS^3 fragment ion spectra of peonidin glycosides.

Supplementary Method S1. Detailed description of the analytical method used for the identification of peonidin glycosides.

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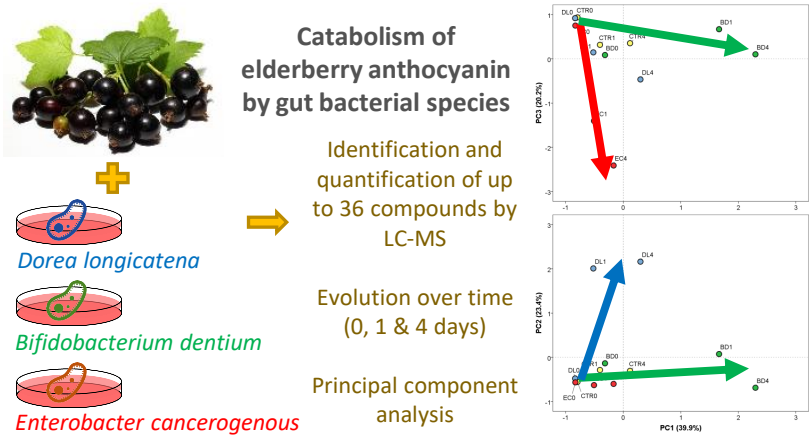
401 **TOC graphic**



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