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1 **Differential catabolism of an anthocyanin-rich elderberry extract by three gut**
2 **microbiota bacterial species**

3 Letizia Bresciani^{1,#}, Donato Angelino^{2,#}, Eugenio Vivas³, Robert Kerby³, Cristina García-Viguera⁴,
4 Daniele Del Rio^{1,2,5}, Federico E. Rey^{3,*}, Pedro Mena^{2,*}

5

6 ¹ *Laboratory of Phytochemicals in Physiology, Department of Veterinary Science, University of*
7 *Parma, 43125 Parma, Italy.*

8 ² *Laboratory of Phytochemicals in Physiology, Department of Food & Drugs, University of Parma,*
9 *43125 Parma, Italy.*

10 ³ *Department of Bacteriology, University of Wisconsin, 53706 Madison, Wisconsin, US*

11 ⁴ *Phytochemistry and Healthy Food Lab, Department of Food Science and Technology, CEBAS-CSIC,*
12 *Campus de Espinardo, 30100 Murcia, Spain.*

13 ⁵ *Microbiome Research Hub, University of Parma, 43124 Parma, Italy*

14 [#] *Equal contribution as first author.*

15

16 ***Correspondence:**

17 Pedro Mena, Human Nutrition Unit, Department of Food & Drugs, University of Parma, Medical
18 School Building C, Via Volturmo, 39, 43125 Parma, Italy. Phone: +39 0521-903841. E-mail:

19 pedromiguel.menaparreno@unipr.it.

20 Federico Rey, Department of Bacteriology, University of Wisconsin-Madison, WI, United States.

21 Phone: +1 608-890-2046 E-mail: ferrey@wisc.edu.

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25 **ABSTRACT**

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

38

39 **KEYWORDS (3 to 5)**

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

41 INTRODUCTION

42 Anthocyanins are a group of phenolic compounds responsible for the red-orange to blue-violet color
43 shades of fruits and vegetables.¹ Cyanidin, peonidin, delphinidin, malvidin, petunidin, and
44 pelargonidin glycosides are the most common anthocyanins distributed in plants.^{2,3} Significant
45 sources of anthocyanins are berries (*i.e.* blueberries, mulberries, blackberries, elderberry,
46 strawberries), red grapes, wine, and pomegranate.⁴ Epidemiological and intervention studies have
47 confirmed that the consumption of anthocyanin-rich foods is associated with a decreased risk of
48 cardiovascular diseases,⁵⁻⁷ an improvement of the antioxidant status,^{8,9} and an amelioration of
49 cognitive performance in impaired individuals.^{10,11} Despite their biological effects, pharmacokinetic
50 studies have demonstrated poor absorption in the upper gastrointestinal tract¹² and a very low
51 bioavailability of anthocyanins, ranging from 0.5% to 1% of the ingested dose.^{13,14} Particularly, it has
52 been showed that they undergo an extensive decomposition in the colonic tract by gut microbiota.¹⁵
53 Intriguingly, it has been suggested that different strains as well as different substituents on the A-,
54 B-, C-rings of the flavylum cation might have a role in driving different breakdown pathways of the
55 anthocyanin structure.¹⁶ The first pioneering work concerning anthocyanin incubation with human
56 fecal inocula indicated very low amounts of the single aglycons after 24-h fermentation.¹⁷
57 Conversely, despite different compounds have been hypothesized to derive from A-ring breakdown,
58 namely phloroglucinol and its aldehyde, resorcinol,^{15,17,18} and ferulic acid,¹⁸ B-ring structure is more
59 prone to the microbiota-mediated transformations. González-Barrio *et al.*¹⁵ hypothesized that the
60 microbial cyanidin B-ring-derived compounds are mainly phenolic acids, as mono- and di-
61 hydroxybenzoic, mono- and di-hydroxyphenylpropionic, and mono- and di-hydroxyphenylacetic
62 acids, among others.¹³ Unfortunately, the use of fecal inocula does not allow clear elucidation of
63 which and how the single gut bacteria strains can access and modify the structure of anthocyanins.
64 Moreover, the metabolic fate of the anthocyanin-derived compounds cannot be drawn by the

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

71

72 **MATERIAL AND METHODS (≤ 500 words)**

73 **Chemicals**

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

82 **Cell Culture**

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

89 Washington University, St. Louis, MO, USA), or left uninoculated as a control, then incubated at
90 37°C. Strains were chosen based on pilot experiments where these strains differentially degraded
91 elderberry anthocyanins into 3,4-dihydroxybenzoic acid (protocatechuic acid, Supplementary Figure
92 S1). In particular, DL and BD produced high and moderate amounts of protocatechuic acid,
93 respectively, while EC degraded the amount of protocatechuic acid in the extract (Supplementary
94 Figure S1). Samples (1.0 mL) were obtained using sterile 75% N₂ / 20% CO₂ / 5% H₂-flushed syringes,
95 injected into 128 µL of 88% formic acid, chilled on ice, centrifuged to pellet cells, then 0.2 µm-filtered
96 and stored at -80°C prior to analysis. Cultures derived from a single flask were assayed for each time
97 point.

98 **Sample preparation and UHPLC-MS/MS and –MSⁿ analyses**

99 Cell media were extracted according to Sala et al.¹⁹, but using acidified methanol (0.1% formic acid)
100 instead of pure methanol. Samples were analyzed for quantification purposes by UHPLC DIONEX
101 Ultimate 3000 equipped with a TSQ Vantage triple quadrupole mass spectrometer (Thermo Fisher
102 Scientific Inc., San Jose, CA, USA), as fully reported in Brindani et al.²⁰ The tentative identification of
103 some compounds was carried out using targeted full MS² experiments with the same instrument
104 and analytical conditions. Quantification was performed with calibration curves of standard
105 compounds, when available. When not available, the metabolites were quantified with the most
106 similar compound in terms of molecular weight and structure.

107 Targeted full MS³ experiments were carried out in order to confirm the identity of some
108 anthocyanins. Experiments were carried out using an Accela UHPLC 1250 equipped with a linear ion
109 trap-mass spectrometer (MS) (LTQ XL) fitted with a heated-electrospray ionization (ESI) probe
110 (Thermo Fisher Scientific Inc.). Analytical details are provided at Supplementary Method S1.

111 **Statistical analysis**

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

116

117 **RESULTS AND DISCUSSION**

118 The set of compounds targeted and/or identified, and their spectrometric characteristics are
119 presented in **Table 1**. Thirty compounds related to the anthocyanin profile of the elderberry extract
120 (compounds **1, 2, 3, 4, 5, and 6**) and to the putative degradation pathway of elderberry anthocyanins
121 (**7-20, 22, 24, 25-29, 33, 34 and 35**) were targeted by considering previous reports.^{15,18,21,22} Eleven
122 out of the 30 compounds were not found in the cultured samples (**11, 12, 14-17, 24, 27, 29, 34, and**
123 **35**), while up to 6 unexpected peaks were observed in the chromatograms (compounds **21, 23, 30-**
124 **32, and 36**). Peak areas for these non-targeted compounds changed in comparison with the
125 uninoculated control medium and depending on the bacteria strains and time points, so they were
126 considered to be potential isomers of the targeted metabolites derived from anthocyanin
127 catabolism. However, although additional commercial standards were run and further *ad hoc* full
128 MS² target experiments were carried out and compared with literature, only compounds **21** and **30**
129 could be identified. Chromatographic and MS² spectra data for these unidentified compounds are
130 reported in **Table 1**. Similarly, additional experiments were carried out in order to fully confirm the
131 presence of peonidin glycosides (**5** and **6**) in the elderberry extract used. Although Olejnik et al.²³
132 reported the presence of these two compounds in an elderberry extract at trace levels, their
133 presence in elderberry contrasted with the typical anthocyanin profile of this berry.²⁴⁻²⁶ Therefore,
134 in order to fully confirm the presence of peonidin derivatives in elderberry, a targeted MS³
135 experiment was conducted. Chromatographic and MS² data for compounds **5** and **6** (Table 1) were

136 in agreement with the evidence reported by Olejnik et al.²³ The fragmentation pattern of both
 137 aglycones (MS² ion fragment at *m/z* 301) accounted for a sole MS³ ion fragment at *m/z* 286
 138 (Supplementary Figure S2), corresponding to the loss of the methyl moiety present on the B-ring of
 139 the peonidin aglycone. These data confirmed the presence of peonidin glycosides in elderberry
 140 extracts.

141

142 **Table 1: Monitored compounds in cell culture samples.**

143

ID	Compound	RT (min)	Parent Ion (<i>m/z</i>)	S- Lens	Quantifier Product Ion (<i>m/z</i>)	CE (V)	Qualifier Product Ion (<i>m/z</i>)	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 (<i>p</i> -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 (<i>m</i> -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

144 Legend: N.I., not identified. These N.I. compounds were observed while analyzing other compounds with the same
145 molecular and fragments ions; they were not identified when subjected to MS² analysis (MS² fragments) and in
146 comparison to other commercially available isomers. They were quantified using the most similar standard according
147 to their molecular weight and their putative structure. N.F., Not Found; STD means the available standard compound
148 used for quantification.

149
150 Quantitative data indicated that cyanidin glycosides accounted for the majority (99%) of the
151 anthocyanins in the elderberry extract (**Table 2**). Considering the 0-day time point, elderberry
152 extract compounds **3** and **4** presented concentrations about 6- and 18-fold higher than compounds
153 **2** and **1**. These data are in agreement with previous studies characterizing the anthocyanin profile
154 of elderberry fruits.^{25,26} Peonidin derivatives accounted for 1% of the anthocyanin profile, in line
155 with previous data.²³ Univariate analyses evidenced several statistically significant differences in the
156 concentration of the 25 compounds detected both among the control medium and the three gut
157 microbiota strains, and among the different collection time points (**Table 2**).

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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 ($\mu\text{mol/L}$)			<i>Enterobacter cancerogenus</i> ($\mu\text{mol/L}$)			<i>Bifidobacterium dentium</i> ($\mu\text{mol/L}$)			<i>Dorea longicatena</i> ($\mu\text{mol/L}$)		
	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.

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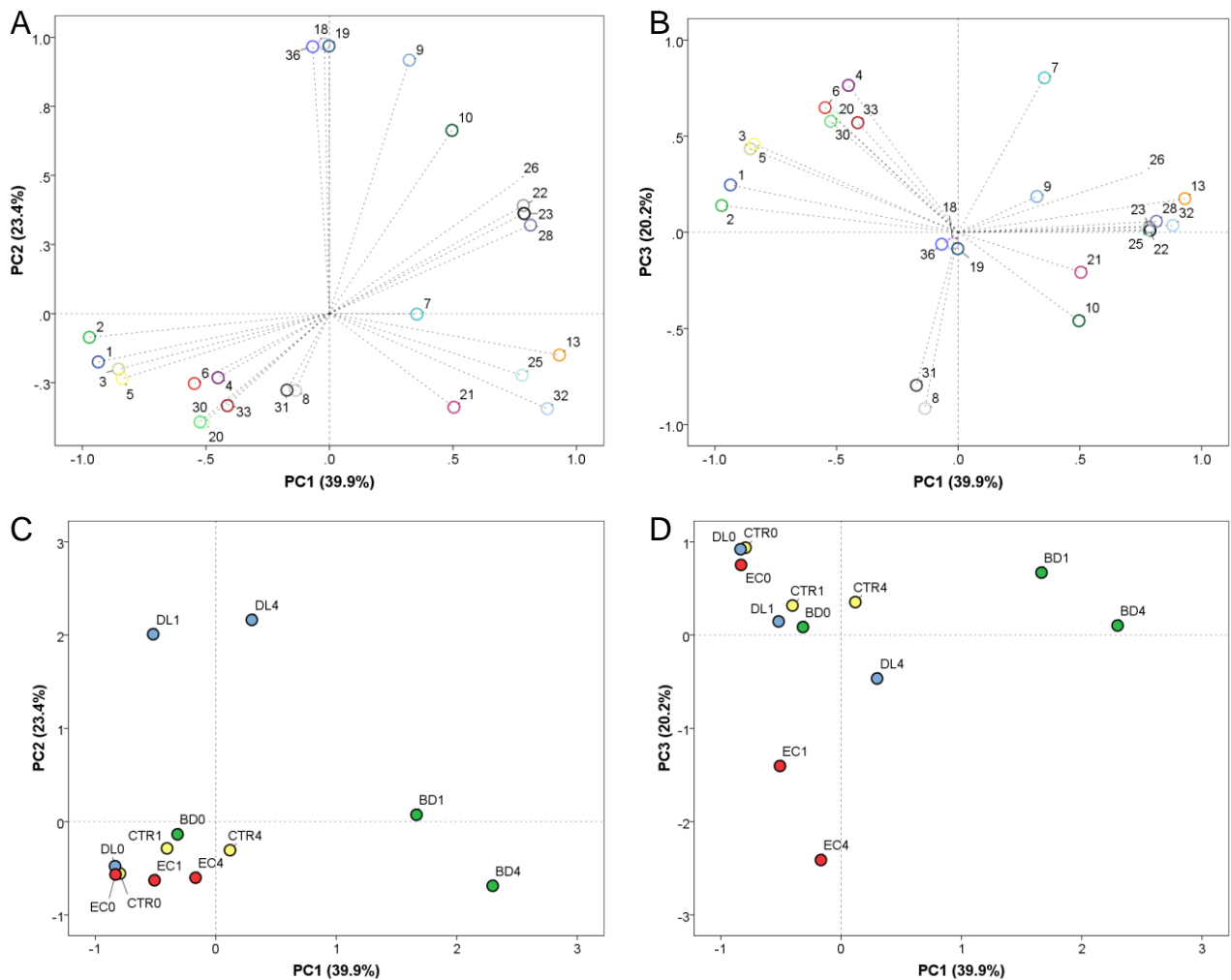
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176 Notably, lower anthocyanin concentrations (for compounds **1**, **2**, **3**, and **5**) were measured in BD
177 cultures compared to control medium, EC, and DL cultures at the 1- and 4-day collection points, as
178 well as for compound **4** in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

186 A multivariate PCA was carried out to better understand the variability in the metabolic pathway of
187 anthocyanin degradation for each gut microbial strain (**Figure 1**). Three principal components (PCs)
188 explained up to 83.5% of the total variability. PC1 accounted for 39.9% of the observed variability
189 and was positively loaded from some phenolics (**13**, **22**, **23**, **25**, **26**, **32**, and **28**), while negatively
190 loaded from most of the anthocyanins (**1**, **2**, **3**, and **5**) (**Figure 1A**, **1B**). PC2 explained 23.4% of the
191 total variability and had positive loadings from protocatechuic acid (**9**), caffeic acid (**18**),
192 methoxybenzaldehyde (**19**), and compound **36** (**Figure 1A**). PC3 accounted for 20.2% of the
193 observed variability and was mostly loaded positively from catechol (**7**) and the anthocyanin
194 sambubioside (**4** and **6**), whereas negatively from pyrogallol (**8**) and compound **31** (**Figure 1B**).
195 Individual scores for each gut bacteria strain and time point revealed interesting insights with regard
196 to the putative degradation pathways of anthocyanins, as compared to the control media (**Figures**
197 **1C** and **1D**). BD-mediated anthocyanin degradation was related to PC1 loads and, thus, to an
198 extensive catabolism of anthocyanins and to increasing amounts of monohydroxylated phenolics
199 (**13**, **25**, and **26**) and C6-C3 backbones (**25**, **26**, and **28**) along the 4-day incubation. DL metabolism,

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



205

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

212

213

214 The microbial degradation of anthocyanins may follow several different pathways, as reported in
215 previous *in vivo* and *in vitro* studies,^{15,18,22} and this aspect highly influences anthocyanin
216 bioavailability. Following the pattern described by González-Barrio *et al.* for raspberry anthocyanins
217 incubated with human fecal suspensions,¹⁵ our results suggested that only EC was able to degrade
218 cyanidin derivatives to yield pyrogallol (**8**) but not catechol (**7**), which was completely degraded.
219 Cyanidin can also be degraded by releasing protocatechuic acid (**9**),¹⁵ which increased along the
220 incubation time in BD and DL, while it decreased in EC. In this sense, De Ferrars *et al.*¹⁸ reported a
221 decreasing trend for protocatechuic acid amounts in fecal samples collected for 2 days after
222 supplementation of human volunteers with ¹³C₅-labelled cyanidin-3-glucoside. Regarding caffeic
223 acid (**18**), a key intermediate of anthocyanin degradation,²² it showed a very significant increment
224 when elderberry extract was incubated with DL, with a moderate increase registered also for BD.
225 Dihydroferulic acid (**28**) also increased notably in presence of the three tested inocula, while ferulic
226 acid (**33**) was not a relevant metabolite. De Ferrars *et al.*¹⁸ evidenced similar results for caffeic acid
227 in fecal samples of volunteers supplemented with ¹³C₅-labelled cyanidin-3-glucoside, but they
228 recovered very high amounts of ferulic acid and not of dihydroferulic acid, although the
229 dehydrogenation of the double bond has been previously hypothesized in the potential microbial
230 metabolic pathways of cyanidin-based anthocyanin.¹⁴ The microbial degradation of cyanidin also led
231 to the formation of phloroglucinaldehyde (**22**),¹⁸ while in our study this compound increased for EC
232 and DL along the 4-day incubation, remaining stable after BD incubation. Moreover,
233 phloroglucinaldehyde has found to be metabolized to methoxybenzaldehyde (**19**),¹⁸ a pathway that
234 was confirmed mainly for DL and just scarcely for BD, while it was not found for EC. Concerning 3,4-
235 dihydroxybenzaldehyde (**10**), an aldehyde putatively derived from protocatechuic acid, it increased
236 for all the tested strains, as did 3-(3'-hydroxyphenyl)propionic acid (**25**).¹⁸

237 To better describe the metabolic transformations carried out by each bacterial strain, efforts were
238 made to identify the unexpected metabolites. Compound **32** ($[M-H]^-$ at m/z 137) was hypothesized
239 to be 2-hydroxybenzoic acid, as it might be formed from phloroglucinaldehyde following the
240 pathway reported by De Ferrars et al.¹⁸ and leading to 2-hydroxy-4-methoxybenzaldehyde
241 formation. This same metabolic pathway may also be linked with the presence of 2-(2'-
242 hydroxyphenyl)acetic acid (**21**). Similarly, compound **36** ($[M-H]^-$ at m/z 181), which was only present
243 in DL samples, was hypothesized to be 2-(3'-hydroxy-4'-methoxyphenyl)acetic acid (aka
244 homoisovanillic acid) since its retention time and fragment ions did not match with those of other
245 isomers expected somehow from cyanidin degradation,^{15,18,22} like 3-(3',5'-
246 dihydroxyphenyl)propionic acid, 3-(2',4'-dihydroxyphenyl)propionic acid, and 3-(4'-
247 hydroxyphenyl)lactic acid. Compound **36** might be related to the degradation pathways yielding
248 other 3'-hydroxy-4'-methoxyphenyl structures (i.e., isovanillic acids).^{15,18,22} Structure for compound
249 **30** ($[M-H]^-$ at m/z 167) did not match with the analytical features of the isomers labelled as putative
250 metabolites (i.e., 2-hydroxy-4-methoxyphenylbenzoic acid, 3-hydroxymandelic acid, 2-(2',4'-
251 dihydroxyphenyl)acetic acid and 2-(3',5'-dihydroxyphenyl)acetic acid), but based on its MS²
252 fragmentation pattern it has been identified as 3-hydroxy-4-methoxy-benzoic acid (isovanillic acid).
253 On contrary, no one of the 2 parent ions $[M-H]^-$ at m/z 151 (**23** and **31**) could be assigned to an
254 isomer of 4-hydroxyphenylacetic acid (**13**) or to a methoxybenzoic acid taking into account their
255 retention times and fragmentation patterns.

256 The interplay between anthocyanin catabolism by the gut microbiota and the microbial modulation
257 caused by anthocyanin consumption is well-known.²⁷ But, despite the different capability to
258 metabolize anthocyanins of the three bacterial species assessed, there is no information linking
259 them to differences in the phenolic metabolism of anthocyanin-rich sources. A previous report²⁸
260 demonstrated that *Clostridium saccharogumia* DSM 17460 and *Eubacterium ramulus* DSM 16296

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

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

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

292

293 **ABBREVIATIONS USED**

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

296

297 **SUPPORTING INFORMATION DESCRIPTION**

298 **Supplementary Table S1.** MML medium composition.

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

301 **Supplementary Figure S2.** Chromatograms and MS³ fragment ion spectra of peonidin glycosides.

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

304

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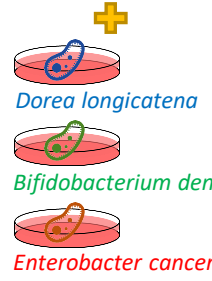
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401 TOC graphic

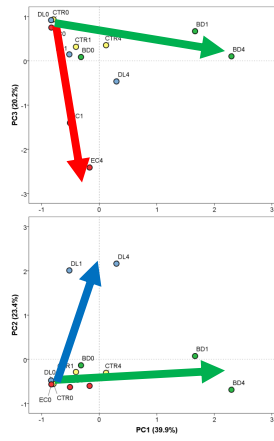


Catabolism of elderberry anthocyanin by gut bacterial species

Identification and quantification of up to 36 compounds by LC-MS

Evolution over time (0, 1 & 4 days)

Principal component analysis



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