RESEARCH ARTICLE

A comparison of the *in vitro* biotransformation of (–)-epicatechin and procyanidin B2 by human faecal microbiota

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The catabolism by human faecal microbiota of (–)-epicatechin (**1**) (2, 3-*cis* stereochemistry) and its dimer pure procyanidin B2 (**2**), has been compared using a static *in vitro* culture model. The catabolites were characterised by LC-MS^n, UV absorption and relative retention time, and quantified relative to standards. No more than ~10% of procyanidin B2 (**2**) was converted to epicatechin (**1**) by scission of the interflavan bond. Five phenolic acid catabolites ($M_r < 290$) were unique to **2**, and ten phenolic acid catabolites ($M_r < 290$) were common to both substrates. The dominant catabolites (≥ 24 h incubation) were 5-(3′-hydroxy phenyl) valeric acid (**9**), 3-(3′-hydroxyphenyl) propionic acid (**10**) and phenyl acetic acid (**12**) (maximum yields 27.4 ± 4.2, 38.2 ± 4.2, 22.7 ± 2.9%, respectively, from **1** and 9.4 ± 1.2, 52.8 ± 2.1, 28.8 ± 1.6%, respectively, from **2**). Substrate **2** was degraded twice as rapidly as **1**. Evidence is presented for the production of previously unreported catabolites of **2** that retain the flavanol A-ring and the C4 → C8 interflavan bond. It was confirmed that catabolism favoured removal of the 4′-hydroxyl rather than the 3′-hydroxyl group and that both β-oxidation and α-oxidation occurred.

Keywords:
Catabolites / Epicatechin / Microbiota / Procyanidin B2

1 Introduction

Polyphenols are of dietary interest because of perceived health-promoting properties [1–3]. Their beneficial effects may in part be due to the metabolites formed in the colon and tissues rather than the parent compounds found in the food [4–11]. Proanthocyanidins are amongst the most abundant (poly)phenols in plants and rich dietary sources include fruits (grapes, peaches, apples, pears and berries), beans, cocoa and beverages such as wine, cider, tea and beer [12]. Proanthocyanidins are oligomers and polymers of the flavan-3-ols [13], particularly (–)-epicatechin.

In vitro studies using crude plant extracts have suggested that proanthocyanidins might have health-promoting properties [14–19], but the methods used do not consider whether large mass compounds are absorbed from the gut, and if so, whether they reach the plasma and tissues untransformed. There is evidence from human intervention studies that cocoa/chocolate consumption may influence some biomarkers related to disease [16, 17, 20] but in these studies it is not possible to assess whether the proanthocyanidins alone are contributing to the effect or whether other components or metabolites are involved.

Procyanidin dimer B2 (epicatechin-(4β → 8)-epicatechin) has been detected in human plasma at 16 ± 5 nM within

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Abbreviations: Rt_R, relative retention time; t_R, retention time

30 min of subjects consuming cocoa [21]. The levels of procyanidin dimer B2 detected in human plasma are lower, sometimes by several orders of magnitude, than the concentrations observed to be effective in many *in vitro* tests of efficacy.

A large fraction of the proanthocyanidins and associated monomers consumed passes to the colon where they are subject to gut microbiotic catabolism, [6, 22, 23] producing phenolic acids (C6–C5, C6–C3 and C6–C1) with varying hydroxylation patterns, [6, 22, 23] and non-phenolic catabolites [22] (perhaps including oxaloacetate, succinate, butyrate and carbon dioxide) [10, 24–26] and unidentified components. The extent to which, for a given flora, the yield and profile of catabolites varies with the substrate, is uncertain. The objective of the present study is to compare the catabolism of (–)-epicatechin with that of its most abundant dimer, procyanidin B2 ((–)-epicatechin-4β→8-(–)-epicatechin). Identified compounds can be developed as biomarkers to determine important inter-individual differences in colonic microbiota metabolism in the future. We have specifically used the word ''catabolism'' for the action of the microbiota and ''fragmentation'' to describe the mass spectral behaviour.

2 Materials and methods

2.1 Chemicals

(–)-Epicatechin was purchased from Extrasynthése, France. Procyanidin B2 and 5-(4′-hydroxy) phenyl valeric acid were synthesized as previously described [40]. 5-Phenyl valeric acid, 5-hydroxy 5-phenyl valeric acid, 3-(3′,4′-dihydroxy phenyl) propionic acid, 3-(3′-hydroxy phenyl) propionic acid, 3-(4′-hydroxy phenyl) propionic acid, 3′,4′-dihydroxy phenyl acetic acid, 3′-hydroxy phenyl acetic acid, 4′-phenyl acetic acid, phenyl acetic acid, 3′,4′-dihydroxy benzoic acid, 3′-hydroxy benzoic acid and 4′-hydroxy benzoic acid were from Fluka Chemical, UK. Bacteriological peptone was from Oxoid, UK and sodium chloride was from Sigma Chemicals, UK. All chemicals were of analytical grade (99%) or the highest grade available.

2.2 Preparation of standards

All the phenolic and phenyl carboxylic acid standards were dissolved in 70% v/v aqueous methanol. The methanolic solutions were sterilized by filtration (Minisart sterile units 0.20 μm).

2.3 Preparation of the substrates

Stock solutions of 5 mM were prepared by dissolving the substrates ((–)-epicatechin or procyanidin B2) into the culture medium and the mixtures were sonicated at ambient temperature for 2 h. To determine the solubility of the substrates the solutions were measured at the wavelength of maximum absorption (275 nm) with a Pye Unicam SP6-450 UV–vis spectrophotometer.

2.4 *In vitro* incubation with human faecal microbiota

The *in vitro* fermentation colonic model was modified [4, 27] and developed according to the needs of the present study. Briefly, the culture broth was prepared by the addition of 0.1% bacteriological peptone and 0.85% sodium chloride dissolved in milli-Q water ($<18.2\Omega$). Steriliszation was achieved by autoclaving at 121°C for 30 min. The broth was deoxygenated for 2 days in anaerobic conditions (10% CO_2, 10% H_2 and 80% N_2) at 37°C and humidified atmosphere. At the end of the deoxygenation period, freshly collected crude faecal microbiota (1 g) from one healthy individual, age 25–30 years, was added in 20 mL broth medium (5%, w/v, faecal suspension) and mixed well. The faecal suspension was left to equilibrate overnight followed by the addition of 9 μmol of substrate. The resulting concentration of the substrate in solution was equal to 0.45 mM, low enough to avoid microbial inhibition [28] and comparatively economical with regard to the use of expensive reagents while permitting reasonably easy detection of substrate and catabolites relative to the background noise. There was a preliminary incubation to define the necessary parameters. For each substrate, there was one experiment incorporating four incubation flasks (one control and three experimental). Each flask was sampled at the specified time points.

Control flasks were used to test the chemical stability of the substrates, by their separate addition to sterile broth or milli-Q water (18.2Ω) without faecal microbiota. Additional control with dead microbiota was used to confirm that changes of the metabolite profile were due to bacterial metabolism and not due to cell binding, or chemical degradation/transformation. The microbiota were inactivated by the addition of chloroform (10 mL/g). A 0.2 M acetate buffer, pH 6.0, was used for adjusting the pH between the ranges 5 and 7 during the incubation periods. The pH was monitored daily. Samples were taken at 3 h intervals from time 0 to 12 h. Additional aliquots were taken at 24 and 48 h.

2.5 SPE of the substrates and catabolites from incubates

The fermentation broths were extracted with a method modified from Coldham *et al.* [29]. Briefly the column used was a Mega Bond Elut C18, 1 g (Varian, UK). The packing was washed with methanol (10 mL) followed by an equal volume of 0.2% v/v formic acid (pH 2.6), to allow equilibration. The fermentation samples (1 mL) were diluted with

1 mL 0.2% formic acid v/v and loaded onto the column. Contaminants were removed by washing with 0.2% v/v formic acid (6 × 5 mL) and the catabolites eluted with methanol (5 mL). The methanolic solutions were evaporated to dryness on a rotary evaporator at 40°C, 130 rpm and 50 mbar. The residues were dissolved in 70% v/v aqueous methanol containing 1 mM ascorbic acid. Authentic standards, where available and stated, were used to confirm identity of specific catabolites detected during chromatographic analysis by LC-ESI-MS^n and to confirm efficient recovery.

2.6 HPLC-ESI-MS^n

The LC equipment (ThermoFinnigan) comprised a Surveyor MS pump, autosampler with 50 μL loop, and a PDA detector with a light-pipe flow cell (recording at 320, 280 and 254 nm, and scanning from 200 to 600 nm). This was interfaced with an LCQ Deca XP Plus mass spectrometer fitted with an ESI source (ThermoFinnigan) and operating in data-dependent, full scan, MS^n mode to obtain fragment ion *m/z*. MS operating conditions (negative ion) were optimised using (–)-epicatechin with a collision energy of 35%, ionisation voltage of 5 kV, capillary temperature of 275°C, sheath gas flow rate of 90 arbitrary units, and auxiliary gas flow rate of 20 arbitrary units. The suitability of these operating conditions to detect the anticipated catabolites was confirmed using a variety of aromatic and phenolic acid standards.

Chromatographic separation was achieved on a 150 × 3 mm column containing Gemini 5 μm phenylhexyl packing (Phenonemex, Macclesfield, UK). Solvent A was water:ACN:glacial acetic acid (975:20:5 v/v/v, pH 2.7) and solvent B was ACN:glacial acetic acid (995:5 v/v). Solvents were delivered at a total flow rate of 300 μL/min. The solvent system started with 96% A and changed to 75% A at 12 min, to 55% A at 40 min and 4% A at 46 min. At 48 min solvent B ran 100% until 53 min and then the system returned to 96% A isocratic until 60 min to re-equilibrate. The total ion current used was between *m/z* 120 and 2000 for the detection of the substrates and catabolites.

2.7 Quantitative analysis

Where possible catabolites were quantified using a calibration curve prepared with a pure commercial standard. Where such standards were not available, catabolites were quantified using a structurally related compound. Calibration curves were prepared at 260 or 280 nm as appropriate using six concentrations between 0.1 and 0.6 mM. The correlation coefficient R^2 and slope for each authentic standard were calculated *via* linear regression as follows: (–)-epicatechin (**1**) (0.995, 4056) also used to quantify **3** and **4**; procyanidin B2 (**2**) (0.996, 598); 3-(3′-hydroxyphenyl) propionic acid (**10**) (0.999, 5672) also used to quantify **6**; phenyl acetic acid (**12**) (0.998, 543). 3-(3′,4′-dihydroxy phenyl) propionic acid (0.993, 6908) (**13**) also used to quantify **5**, **7** and **8**. 3-(4′-hydroxyphenyl) valeric acid (0.997, 38737) also used to quantify **9**.

2.8 Statistical analysis

Results are shown as mean ± SD. A two-tailed unpaired *t*-test assuming unequal variances was used to make comparisons when necessary.

3 Results

3.1 Recovery of standards from microbial medium

The microbial medium was spiked with a selection of standards in order to assess the recovery of typical catabolites. The recoveries were calculated as 92 ± 3.3% for (–)-epicatechin, 86 ± 1.8% for 3-(3′-hydroxy phenyl) propionic acid, 88 ± 2.6% for 3-(3′,4′-dihydroxy phenyl) propionic acid, 78 ± 3.2% for 3′,4′,5′-trihydroxy benzoic acid and 82 ± 2.3% for 3′-hydroxy phenyl acetic acid.

3.2 LC-MS of authentic standards

Table 1 summarizes retention time (t_R) and mass fragmentation data for a range of phenolic acids known or likely to be found as microbial catabolites of 1 and/or 2. From these data it was observed that 4′-hydroxy derivatives elute earlier than 3′-hydroxy (relative retention time (Rt_R) = 1.14 ± 0.11 for $N = 4$). Similarly, 3′,4′-dihydroxy derivatives elute earlier than their 3′-hydroxy analogues (Rt_R 1.24 ± 0.09 for $N = 4$) and their 4′-hydroxy analogues (Rt_R = 1.41 ± 0.16 for $N = 4$).

3.3 Characterisation of the catabolites common to (–)-epicatechin and procyanidin B2

Substrates and, where possible, catabolites were identified in the chromatograms by the use of authentic standards. In the absence of authentic standards, structural assignments were made from a critical examination of the molecular mass, mass fragmentation data, UV spectrum and Rt_R. It was not possible to define the chiral properties of the catabolites in this study. While this study was in progress it has been reported that gut microbiota can convert (+)-catechin to a mixture of (+)-epicatechin and (–)-epicatechin [30] and the two epicatechin enantiomers would not have been resolved on the reversed-phase column packing used in this study. Accordingly, other than as a substrate at zero time, peak (**1**) might be

Table 1. LC-ESI-MS^n fragmentation data for authentic standards

Name	t_R/min[a]	Parent ion	MS^2 base peak	MS^2 Secondary ions		MS^3 base peak	MS^3 secondary ions	
		m/z	*m/z*	*m/z*	Intensity	*m/z*	*m/z*	Intensity
3′,4′-Dihydroxy benzoic acid	9.39	153.0	109.0	153.0	10	n.d.[b]	n.d.	n.d.
3′-Hydroxy benzoic acid	12.91	137.1	93.1	137.0	5	n.d.	n.d.	n.d.
4′-Hydroxy benzoic acid	11.62	137.1	93.1	137.0	5	93.0	59.0	2
3′,4′-Dihydroxy phenyl acetic acid	9.15	167.0	123.1	167.0	2	95.0	123.0	80
3′-Hydroxy phenyl acetic acid	13.35	151.0	107.0	93.0	15	93.0	n.d.	n.d.
4′-Hydroxy phenyl acetic acid	11.94	151.0	107.0	n.d.	n.d.	n.d.	n.d.	n.d.
Phenyl acetic acid	18.20	135.0	91.0	n.d.	n.d.	n.d.	n.d.	n.d.
5-Phenyl valeric acid	32.38	177.1	159.1	177.0	5	141.1	n.d.	n.d.
5-Hydroxy 5-phenyl valeric acid	19.01	193.1	175.1	101.0	80	131.1	157.1	60
5-(4′-Hydroxy) phenyl valeric acid	21.52	193.0	175.0	193.0	80	175.0	157.1	30
3-(3′,4′-Dihydroxy phenyl) propionic acid	9.02	167.0	123.1	n.d.	n.d.	n.d.	n.d.	n.d.
3-(3′-Hydroxy phenyl) propionic acid	15.20	165.0	121.0	119.1	30	119.0	121.0	80
3-(4′-Hydroxy phenyl) propionic acid	14.31	165.0	121.0	93.0	35	93.0	121.0	65

a) Identifications based on t_R and MS*n* data obtained with negative ionisation.
b) Not detected under these conditions.

a mixture of enantiomers and therefore will henceforward be referred to as epicatechin rather than (–)-epicatechin. A similar uncertainty applies to the chirality of the C-ring-opened catabolites (3 and 4) and the C_6–C_5 catabolites that retain a side chain hydroxyl group (7 and 16). For this reason, we make no chiral designation when referring to these catabolites. In addition, no catabolites were formed in the absence of bacteria or with inactivated bacteria.

(–)-Epicatechin (**1**) and procyanidin B2 (**2**) were incubated (37°C) with freshly isolated human faecal microbiota (5% w/v) and extracts were analyzed by LC-MS. The metabolic profiles obtained from the anaerobic incubations of **1** and **2** were similar, revealing eleven abundant catabolites, all of which were absent from the control samples (data are not shown). UV chromatograms of **1**, **2** and their catabolites are shown in Figs. 1A and B at designated time intervals up to 24 h.

The availability of authentic standards facilitated the identification of several catabolites observed at later time points ($\geq$11 h incubation) (Fig. 1B), as 3-(3′-hydroxy phenyl) propionic acid (**10**) and 3′-hydroxy phenyl acetic acid (**11**), phenyl acetic acid (**12**) and 3-(3′,4′-dihydroxy phenyl) propionic acid (**13**) (Fig. 2). The unequivocal identification of **13** demonstrates the relative stability of the B-ring to catabolism compared with the A-ring of **1**, as previously reported [8, 9]. Similarly, the unequivocal identification of **10** and **11** and the absence of their 4′ analogues establishes that the 4′-hydroxyl group is removed more readily than the 3′-hydroxyl group as previously shown [6, 8, 31, 32].

The fragmentation behaviour of catabolites **3** and **4** was identical with that of (–)-epicatechin (**1**) (Table 2) with losses of 44 and 42 amu during MS^2 and MS^3, respectively. These data suggest that **3** and **4** retain the structural features that determined the fragmentation sequence for **1**. However, the ions obtained from **3** were always 2 amu higher than the equivalent ions from **1**. Consequently **3** was identified as 1-(3′,4′-dihydroxy phenyl)-3-(2″,4″,6″-trihydroxy phenyl) propan-2-ol (Fig. 2). In a previous study [33], a similar catabolite has been defined as the (*2S*)-isomer. Catabolite **4** produced ions 16 amu less than **3** suggesting that it contained one less hydroxyl group than catabolite **3**. The fragment ions of $[M–H^+]^-$ *m/z* 275 (Table 2) indicate that the hydroxyl group has been lost from the B-ring but does not define whether it is the hydroxyl group at position 3′ or 4′.

Two catabolites that eluted at 14.9 and 18.5 min produced $[M–H]^-$ parent ions at *m/z* 207 and 191, respectively, fragmented identically (Table 2) suggesting two similar compounds differing by one oxygen atom. The Rt_R of 1.24 is consistent with the faster eluting peak being a 3′,4′-dihdroxy compound and the slower eluting peak being the 3′-hydroxy analogue. The $[M–H]^-$ MS^2 base peak at *m/z* 163 obtained from the $[M–H]^-$ parent ion at *m/z* 207 is typical of the 5-(3′,4′-dihydroxy phenyl)-γ-valerolactone, based on data in the literature [30, 34]. Consequently and by analogy of their molecular mass and fragmentation, these were assigned as 5-(3′,4′-dihydroxy phenyl)-γ-valerolactone (**5**) and 5-(3′-hydroxy phenyl)-γ-valerolactone (**6**) (Fig. 3).

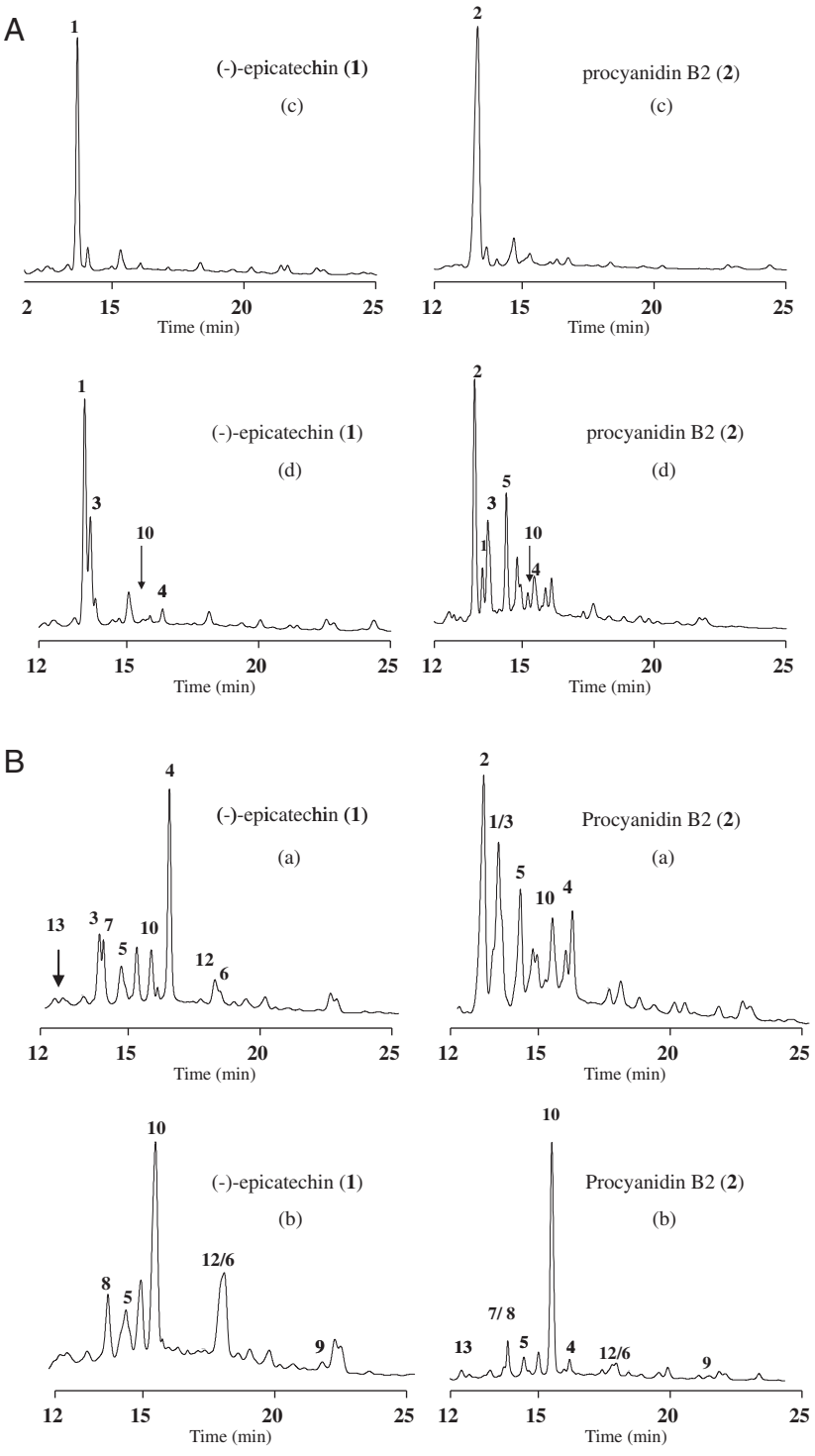

Figure 1. (A) Time-dependent UV chromatograms (260–280 nm) after anaerobic incubation (37°C) of **1** and **2** with human faecal microbiota (5% w/v). (c) Fermentation time 0 h: substrates **1** and **2** are shown. (d) Fermentation time 6 h: formation of catabolites **3**, **4**, **5** and **10** with both substrates **1** and **2**. (B) Time-dependent UV chromatograms (260–280 nm) after anaerobic incubation (37°C) of **1** and **2** with human faecal microbiota (5% w/v). (a) Fermentation time 12 h: Catabolites **6**, **7**, **10**, **12** and **13** have been formed with substrate **1**, but not with **2**. (b) Fermentation time 24 h: formation of catabolites **6**, **7**, **10**, **12** and **13** with substrate **2** and formation of catabolites **8** and **9** for both anaerobic incubations with substrates **1** and **2**.

Catabolite **9** ($M_r = 194.06$) had fragmentation behaviour (Table 2) identical to the authentic standard 5-(4′-hydroxy phenyl) valeric acid ($M_r = 194.06$) but eluted approximately 1 min later. This difference in retention ($Rt_R = 1.09$) suggests that catabolite **9** is 5-(3′-hydroxy phenyl) valeric acid. It has previously been observed that 4′ hydroxyl group is removed more extensively by the gut microbiota than the 3′ hydroxyl group [6, 8, 31, 32].

A catabolite was detected (t_R = 14.35 min $[M–H]^-$ at m/z 209) that lost 18 and 44 amu at MS^2 and MS^3, respectively, exactly as observed for catabolite **9**, thus suggesting that it may be an analogue containing one extra oxygen. The

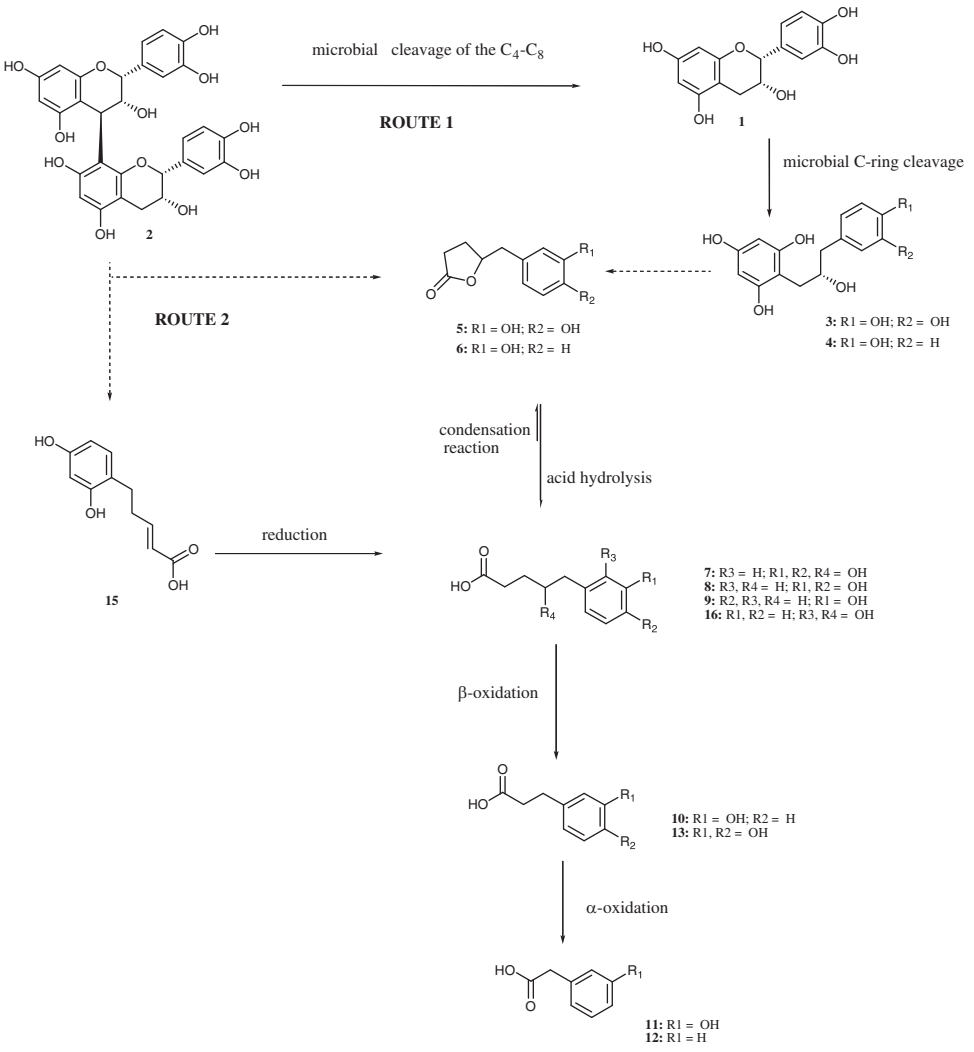

Figure 2. Proposed catabolic pathways for the formation of small molecular weight phenolic and phenyl carboxylic acid derivatives from the anaerobic incubation (37°C) of (–)-epicatechin (**1**) and procyanidin B2 (**2**) with human faecal microbiota (5% w/v) *in vitro*.

difference in retention between this unknown and the slower eluting 5-(4′-hydroxy phenyl) valeric acid standard ($Rt_R = 1.59$) are consistent with the faster eluting unknown being 5-(3′,4′-dihydroxy phenyl) valeric acid (**8**).

A catabolite with parent ion at $[M–H]^-$ *m/z* 225 eluted at 14.22 min. This mass suggests a C_6–C_5 phenolic acid with three hydroxyl groups, and structure **7** is the only such catabolite likely to form from **1** and **2** (Fig. 2). The mass observed, the fragmentation (Fig. 4) and elution before **8** are consistent with this structure but in the absence of appropriate standards or close analogues the assignment as 5-(4-hydroxy)-(3′,4′-dihydroxy) phenyl valeric acid is tentative.

3.4 Characterisation of the low molecular mass catabolites unique to procyanidin B2

Five small molecular mass catabolites were identified solely in the procyanidin B2 incubations. As far as we are aware, such catabolites have not previously been reported. Accordingly an attempt has been made to characterise them as described in the previous section, but in the absence of appropriate standards, definitive identification is not possible. All had a λ_{max} near 280 nm, indicating that they each contain at least one intact aromatic ring. Their apparent absence from the (–)-epicatechin incubations suggests that they might retain the inter-monomer (C4→C8) bond and the aromatic ring might therefore be a *meta* substituted A-ring rather than the *ortho*-dihydroxy B-ring associated with the catabolites discussed above. Theoretically the side chain of such *meta*-substituted catabolites could be formed either by a degradation of the other A-ring or from the C-ring and B-ring of the other flavanol moiety.

Four catabolites were detected ($1 \times M_r = 208$ and $3 \times M_r = 210$) for which C_6–C_5 structures based on an intact A-ring could be proposed. One of these ($t_R = 14.6$ min. $[M–H]^-$ *m/z* 207) lost 108 amu at MS^2 and might be 5-(2′,4′-dihydroxy) phenyl-2-ene valeric acid **15** or its 2′6′-dihydroxy

Table 2. LC-ESI-MS^n fragmentation data for small molecular weight catabolites detected during (–)-epicatechin (**1**) and procyanidin B2 (**2**) anaerobic incubation (37°C) with human fecal microbiota (5% w/v)

No.	t_R/min[b)]	Parent ion	MS^2 Base peak[a)]		MS^2 secondary ions						MS^3 Base peak		MS^3 secondary ions	
		m/z	*m/z*	Intensity	*m/z*	Intensity	*m/z*	Intensity	*m/z*	Intensity	*m/z*	Intensity	*m/z*	Intensity
1	13.85	289.0	245.0	100	205.1	40	179.1	20	161.2	5	203.0	100	245.1	40
2	13.53	577.0	425.0	100	407.0	40	451.0	25	289.1	15	407.0	100	n.d.[c)]	n.d.
3	14.06	291.1	247.1	100	167.1	25	205.1	20	123.1	15	205.0	100	247.1	90
4	16.74	275.1	231.1	100	217.1	65	191.0	30	125.0	20	189.0	100	171.2	60
5	14.9	207.1	163.1	100	189.1	45	n.d.	n.d.	n.d.	n.d.	163.1	100	n.d.	n.d.
6	18.52	191.1	147.1	100	191.0	55	173.2	25	107.1	10	129.0	100	n.d.	n.d.
7	14.22	225.1	181.1	100	165.0	30	225.1	15	n.d.	n.d.	181.0	100	n.d.	n.d.
8	14.35	209.1	191.1	100	147.0	60	101.0	40	n.d.	n.d.	147.1	100	n.d.	n.d.
9	22.71	193.1	175.1	100	149.0	15	193	10	n.d.	n.d.	157.0	100	175.0	20
10	16.00	165.0	121.1	100	119.1	60	n.d.	n.d.	n.d.	n.d.	121.0	100	n.d.	n.d.
11	15.07	151.0	107.0	100	92.9	15	121.0	5	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
12	18.47	135.0	91.0	100	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
13	12.31	181.0	137.0	100	119.0	15	n.d.	n.d.	n.d.	n.d.	137.0	100	n.d.	n.d.

Compounds are as follows: **1**, (–)-epicatechin; **2**, procyanidin B2; **3**, 1-(3′,4′-dihydroxy phenyl)-3-(2″,4″,6″-trihydroxy phenyl) propan-2-ol; **4**, 1-(hydroxy phenyl)-3-(2″,4″,6″-trihydroxy phenyl) propan-2-ol; **5**, 5-(3′,4′-dihydroxy phenyl)-γ-valerolactone; **6**, 5-(3′-hydroxy phenyl)-γ-valerolactone; **7**, 5-(4-hydroxy)-(3′,4′-dihydroxy) phenyl valeric acid (tentative); **8**, 5-(3′,4′-dihydroxy phenyl) valeric acid; **9**, 5-(3′-hydroxy phenyl) valeric acid; **10**, 3-(3′-hydroxy phenyl) propionic acid; **11**, 3′-hydroxy phenyl acetic acid; **12**, phenyl acetic acid; **13**, 3-(3′,4′-dihydroxy phenyl) propionic acid.

a) Peak numbers and t_R refer to peaks in Figs. 1a and b.

b) Identifications based on t_R and MS_n data obtained with negative ionization.

c) Not detected under these conditions.

isomer if the C-ring had opened. These might form through route 2 as shown in Fig. 2. The fragmentation mechanism is shown in Fig. 5.

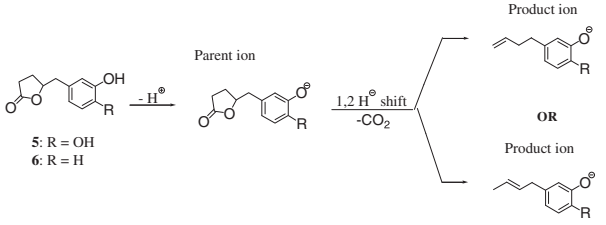

Figure 3. Proposed mechanism for the fragmentation of **5** (R=OH) and **6** (R=H) *via* 1,2 hydride-shift in the lactone ring generating both allyl and vinyl phenoxide anions respectively. For MS^n data and t_R refer to Table 2.

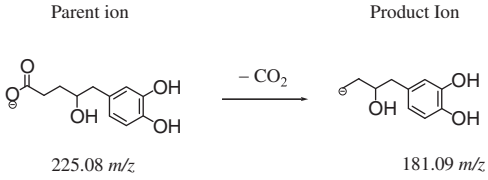

Figure 4. Proposed fragmentation mechanism for **7** during MS^2 *via* decarboxylation from the end of the side chain. For MS^n data and t_R refer to Table 2.

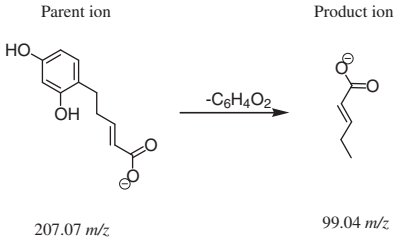

Figure 5. Proposed fragmentation mechanism for **15** during MS^2 (loss of 108 amu). For MS^n data and t_R refer to Table 3.

Three isomers with parent ions $[M–H]^-$ at *m/z* 209 eluted at 7.10 min (**16**), 15.84 min (**17**) and 17.77 min (**18**). Catabolites **16** (assigned as 5-(3′,4′-dihydroxy phenyl) valeric acid) and **8** fragmented identically losing 18 amu at MS^2 and 44 amu at MS^3 (Fig. 6), suggesting similar structures that differ by some feature that significantly affects the hydrophilicity ($Rt_R = 2.02$). Catabolite **18** also lost 18 amu at MS^2 but 56 amu at MS^3, whereas isomer **17** lost a distinctive 88 amu at MS^2 suggesting progressively greater structural differences compared with **8** and **16**.

Catabolite **14** had the same molecular ion $[M–H]^-$ *m/z* 291 and the same MS^2 ion as **3** but then lost 28 amu at MS^3 compared with 42 amu for **3**. Catabolite **14** was more hydrophilic than **3** ($Rt_R = 1.53$). The structures of **14**, **17** and **18** were not investigated further.

3.5 Quantification of the substrates (–)-epicatechin and procyanidin B2 and their common catabolites

Procyanidin B2, (–)-epicatechin (or epicatechin as appropriate) and ten other catabolites were quantified by their UV absorption relative to authentic standards. The changes in concentration with time of incubation are shown in Figs. 7 and 8 and the calculated molar mass recoveries in Tables 4 and 5. Total molar mass recoveries ranged between 77 and 103% for (–)-epicatechin (**1**) and between 81 and 110% for procyanidin B2 (**2**).

Maximum molar mass recoveries (%) for each catabolite, following the anaerobic incubation of **1** and with ascending sequence, were as follows: catabolite **3** (23.7 ± 4.1%) at 6 h, catabolites **4** > **7** (11.8 ± 0.9 and 10.8 ± 0.7%, respectively) at 12 h, catabolites **5** > **6** > **8** > **13** (20.8 ± 0.5, 15.9 ± 2.1, 4.9 ± 0.6 and 4.5 ± 0.6%, respectively) at 24 h and catabolites **10** > **9** > **12** (38.3 ± 4.2, 27.4 ± 4.2 and 22.7 ± 2.9%, respectively) at 48 h. Similarly, following anaerobic incubation of **2**, maximum molar mass recoveries (%) for each catabolite with ascending sequence were: catabolites **1** (6.2 ± 0.6%) at 6 h, catabolites **5** > **3** > **4** (27.1 ± 2.3, 12.6 ± 0.8 and

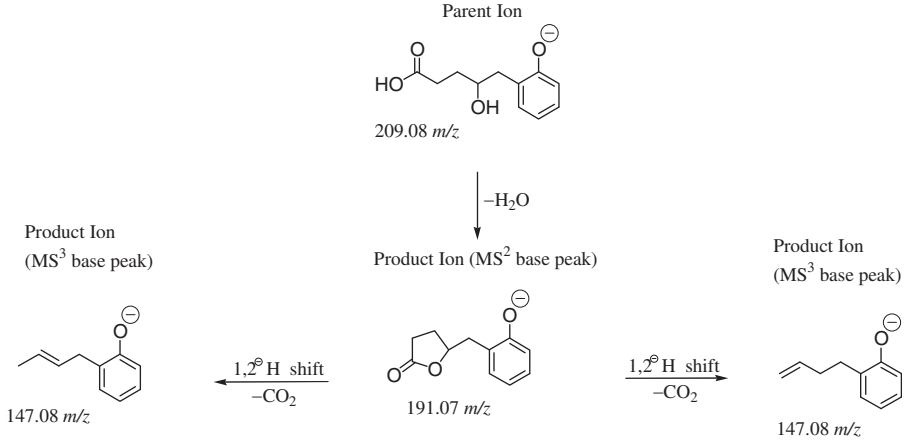

Figure 6. Proposed fragmentation mechanisms for **16**, during MS^2 and MS^3 *via* condensation reaction and 1,2 hydride-shift, respectively. For MS^n data and t_R refer to Table 3.

Table 3. LC-ESI-MS^n fragmentation data for small molecular weight catabolites detected during procyanidin B2 (**2**) anaerobic incubation (37°C) with human fecal microbiota (5% w/v)

No.	t_R/min[a)]	Parent Ion	MS^2 base peak		MS^2 secondary ions						MS^3 base peak		MS^3 secondary ions	
		m/z	*m/z*	Intensity	*m/z*	Intensity	*m/z*	Intensity	*m/z*	Intensity	*m/z*	Intensity	*m/z*	Intensity
14	9.16	291.0	247.2	100	273.1	20	223.1	18	163.0	18	219.3	100	189.1	77
15	14.16	207.0	99.0	100	163.1	80	107.1	70	189.0	60	n.d.[b)]	n.d.	n.d.	n.d.
16	7.10	209.0	191.0	100	147.0	15	129.0	10	n.d.	n.d.	147.0	100	n.d.	n.d.
17	15.84	209.0	121.0	100	165.1	10	209.2	5	99.7	2	n.d.	n.d.	n.d.	n.d.
18	17.77	209.0	191.0	100	135.1	45	209.1	15	147.0	10	135.1	100	191.3	25

Compounds are: **14**, **17** and **18**, not positively identified, see text; **15**, 5-(2′,4′-dihydroxy) phenyl-2-ene valeric acid (tentative); **16**, 5-(3′,4′-dihydroxy phenyl) valeric acid (tentative).
a) Identifications based on retention times (t_R) and MS*n* data obtained with negative ionisation.
b) Not detected under those conditions.

Table 4. Molar mass recoveries for (–)-epicatechin (**1**) and related catabolites (%)

	0 (h)	3 (h)	6 (h)	9 (h)	12 (h)	24 (h)	48 (h)
1	90.4±8.2	75.8±4.2	62.7±8.1	28.3±1.2	n.d.[a)]	n.d.	n.d.
3	n.d.	1.4±0.08	23.7±4.1	9.6±1.2	13.8±1.1	n.d.	n.d.
4	n.d.	n.d.	5±0.6	2.8±0.2	11.8±0.9	n.d.	n.d.
5	n.d.	n.d.	n.d.	15.8±0.7	18.9±0.9	20.8±0.5	4.1±0.5
6	n.d.	n.d.	n.d.	1.1±0.1	3.3±0.4	15.9±2.1	5.7±0.5
7	n.d.	n.d.	n.d.	4.3±0.5	10.8±0.7	2.4±0.1	3.3±0.4
8	n.d.	n.d.	n.d.	n.d.	3.8±0.9	4.9±0.6	n.d.
9	n.d.	n.d.	n.d.	n.d.	n.d.	11.2±0.7	27.4±4.2
10	n.d.	n.d.	n.d.	11.4±1.1	13.8±0.4	26.8±3.8	38.3±4.2
12	n.d.	n.d.	n.d.	8.2±0.6	9.8±1.2	15.6±0.7	22.7±2.9
13	n.d.	n.d.	n.d.	n.d.	3.9±0.3	4.5±0.6	1.3±0.1
Total	90.4	77.2	91.4	81.5	89.9	102.1	102.8

Mass recoveries (%) were calculated from Fig. 7.
a) Not detected.

Table 5. Molar mass recoveries for procyanidin B2 (**2**) and related catabolites (%)

	0 (h)	3 (h)	6 (h)	9 (h)	12 (h)	24 (h)	48 (h)
1	n.d.[a)]	6.2±0.6	6.4±0.8	3.6±0.8	2.9±0.4	n.d.	n.d.
2	97.2±12.9	69.6±2.6	58.4±7.8	54.8±2.6	34.2±3.1	n.d.	n.d.
3	n.d.	5.5±0.4	11.8±0.9	6.1±0.6	12.6±0.8	n.d.	n.d.
4	n.d.	n.d.	2.3±0.3	2.6±0.4	8.1±0.5	1.1±0.08	n.d.
5	n.d.	n.d.	17.3±4.5	15.6±2.2	27.1±2.3	8.7±2.3	n.d.
6	n.d.	n.d.	n.d.	n.d.	n.d.	3.4±0.2	n.d.
7	n.d.	n.d.	n.d.	n.d.	n.d.	2.3±0.5	1.9±0.4
8	n.d.	n.d.	n.d.	n.d.	n.d.	3.2±0.3	8.1±0.5
9	n.d.	n.d.	n.d.	n.d.	n.d.	4.1±0.8	9.4±1.2
10	n.d.	n.d.	9.7±1.2	18.4±0.9	24.6±3.6	52.8±2.1	48.9±3.4
12	n.d.	n.d.	n.d.	n.d.	n.d.	6.9±0.8	28.8±1.6
13	n.d.	n.d.	n.d.	n.d.	n.d.	1.9±0.1	1.1±0.06
Total	97.2	81.3	105.9	101.1	109.5	84.4	98.2

Mass recoveries (%) were calculated from Fig. 8.
a) n.d.: not detected.

8.1 ± 0.5%, respectively) at 12 h, **10** > **6** > **7** > **13** (52.8 ± 2.1, 3.4 ± 0.2, 2.3 ± 0.5 and 1.9 ± 0.1%, respectively) at 24 h and finally catabolites **12** > **9** (28.8 ± 1.6 and 9.4 ± 1.2%, respectively) at 48 h. The lowest total recovery was observed at 3 h and the deficit might in part be explained by the formation of non-aromatic catabolites such as oxaloacetate and carbon dioxide [35]. In the case of **2**, the ''dimeric'' metabolites also account for some of this apparent deficit at 3 h but because their yields were low and their chromatographic resolution was poor, they were not quantifiable by UV detection. Where over 100% total recovery was calculated (≥ 6 h), it may be partly because of experimental error, partly because of using one standard to quantify several related compounds that may differ slightly in their molar absorbance and partly due to the possible formation of more than one product from the same substrate.

4 Discussion

This study is the first comparison of the catabolism by human-associated faecal microorganisms of a monomeric flavanol, (–)-epicatechin (**1**), and an associated dimer, procyanidin B2 (**2**). Substrate loss and catabolite formation were quantified and catabolites characterised by ion trap LC-MS. Three classes of catabolite were detected: (i) catabolites with $M_r < 290$ formed from both substrates; (ii) catabolites with $M_r < 290$ formed uniquely from (**2**) and (iii) catabolites with $M_r > 290$ formed from (**2**). Catabolites in categories (i) and (ii) are discussed here: those in category (iii) will be reported separately. Of the ten catabolites in category (i), several have been reported previously in studies using various flavanols or proanthocyanidins and either animal-associated faecal microorganisms (**4**, **6**, **9**–**13**) [8, 9], single microbial strains of faecal origin (**3**, **4**) [32], human-associated microorganisms (**3**–**6**, **9**–**13**) [6, 34, 36, 37] or have been found in human plasma and/or urine after absorption and mammalian metabolism (**10**) [35]. Some (**10**–**13**) have been detected in the faecal water of humans consuming a diet that contained a wide range of polyphenols in addition to flavanols [37].

Some catabolites reported in these previous studies were not detected currently. For example, 5-phenyl-γ-valerolactone and 5-(4′-hydroxyphenyl)-γ-valerolactone, similar to catabolites **5** and **6** reported here, have been detected in animal [31, 38, 39], human [32, 36] and *in vitro* studies [34]. The absence of 4′-hydroxyphenolic acid catabolites in the present study is proven since pure standards were available. Although previous reports of these ''missing'' catabolites might suggest misidentification when pure standards are not available, such differences more probably reflect variations in the composition and biochemical competence of individual microbiota.

It has been reported that microbial metabolism of (+)-catechin proceeds through (+)-epicatechin [30], a conversion that requires inversion of the chiral centre at C2. In this study, (+)-catechin was not investigated but there was no evidence to suggest the formation of (–)-catechin from (–)-epicatechin (**1**).

As far as we are aware, catabolite **7**, tentatively identified as 5-(4-hydroxy)-(3′,4′-dihydroxy) phenyl valeric acid, has not previously been reported. Confirmation must await synthesis of the pure substance in the correct chiral form. The 4*S* isomer would be expected from either (+)-catechin or (+)-epicatechin, whereas the 4*R* isomer would be expected from (–)-catechin or (–)-epicatechin.

A prominent feature of flavanol catabolism is the progressive β-oxidation of the C_6–C_5 catabolites to C_6–C_3 and C_6–C_1. In this study, using pure substrates, we have demonstrated also an α-oxidation generating a C_6–C_2 catabolite, 3′-hydroxy phenyl acetic acid (**11**). Such α-oxidations are a recognised feature of flavonol catabolism and apart from a study reported recently [34],] these had not been observed with pure flavanols but only with crude proanthocyanidin-rich preparations [6] that might have contained flavonol contaminants.

4.1 Low molecular mass catabolites derived from both (–)-epicatechin and procyanidin B2

The initial rate of (–)-epicatechin catabolism was 20 ± 4.6 μmol/h (~4.5%/h). Procyanidin B2 (**2**) was catabolised more rapidly (40 ± 7.8 μmol/h, ~9%/h) to yield the same ten catabolites and epicatechin of undetermined chirality. Epicatechin was not present as a contaminant at zero time but either (+)-epicatechin, (–)-epicatechin, or a mixture, was clearly present in the procyanidin B2 (**2**) incubations up to 12 h and must have formed *via* cleavage of the C4 → C8 interflavan bond. The initial net production rate was 7.0 ± 0.4 μmol/h, equivalent to a gross production rate of 7.3 ± 0.3 μmol/h assuming that the (%)-epicatechin produced also was catabolised at ~4.5%/h.

If cleavage of the interflavan bond was the sole route of catabolism then the net production rate for epicatechin should have been some 80 μmol/h and the gross rate approximately 84 μmol/h. The observed rate was less than 9% of the predicted rate, indicating that catabolism by cleavage of the interflavan bond is unlikely to have accounted for more than 10% of the total catabolism.

The trapezoidal area under the curve values for the time period zero to 48 h (Figs. 7 and 8) were calculated as estimates of the yields of catabolites **3** and **4** from both substrates. The production of both catabolites was significantly lower ($p < 0.05$) from procyanidin B2 (**2**) being approximately half the yields from **1**, consistent with the above argument.

It has been proposed previously that the cleavage of the C4 → C8 link is a major route for procyanidin B3 catabolism, generating free (+)-catechin [8], which then could degrade in the same way as the monomer [7–9, 32]. Production of (+)-catechin was not observed in that study but that may have been due to the lack of sampling at the early time

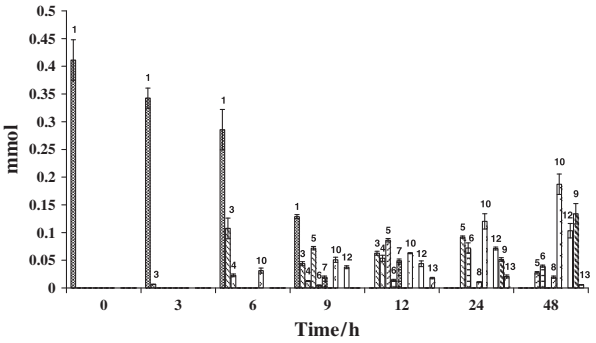

Figure 7. Quantitative analysis for substrate **1** and related catabolic derivatives during 48 h of anaerobic incubation (37°C) with human faecal microbiota (5% w/v). Results are a mean ± SD for three different anaerobic incubations.

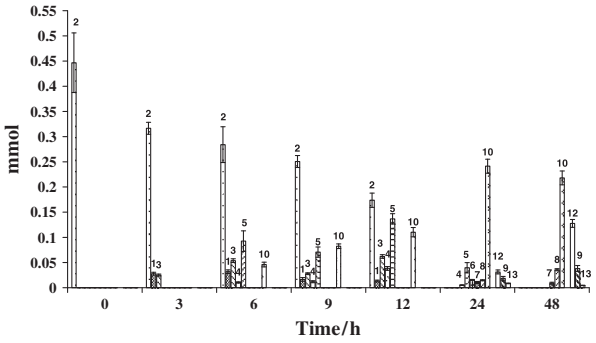

Figure 8. Quantitative analysis for substrate **2** and related catabolites during 48 h of anaerobic incubation (37°C) with human faecal microbiota (5% w/v). Results are a mean ± SD for three different anaerobic incubations.

points such as used in the current study. Significant cleavage of the interflavan bond has also been reported in a study of the human microbiota catabolism of mixed procyanidin dimers (B1, B2, B3 and B4) [34], again pointing to the marked inter-person variation in the competence of the colonic microbiota.

4.2 Low molecular mass catabolites detected solely from procyanidin B2 incubations

For the first time catabolites small enough to have been formed from (–)-epicatechin (**1**) have been found that are unique to the incubation of procyanidin B2 (Table 3). It is suggested that some of these might be novel *meta*-hydroxylated phenols containing an intact A-ring and the associated C4→C8 interflavan bond. Two of these (**15** and **16**) were tentatively identified as 5-(2′,4′-dihydroxy) phenyl-2-ene valeric acid, and 5-(3′,4′-dihydroxy phenyl) valeric acid respectively, but confirmation of these identifications must await synthesis of relevant standards and full characterisation of the other four requires further investigation. These catabolites were most obvious at ~3 h where the total molar recovery is comparatively low.

4.3 Quantitative analysis of common catabolites produced after the incubation of (–)-epicatechin or procyanidin B2 with human faecal microbiota

The relative mass yields can be estimated at specific time points (Figs. 7 and 8) and it is clear that beyond 24 h catabolites **9**, **10** and **12** are dominant (maximum yields 27.4 ± 4.2, 38.2 ± 4.2, 22.7 ± 2.9%, respectively, from **1** and 9.4 ± 1.2, 52.8 ± 2.1, 28.8 ± 1.6%, respectively, from **2**). While this observation suggests that *in vivo* these particular catabolites might be of greater interest with regard to potential biological effects on the host, other factors may modify this. For example, person to person variation in the composition of the microbiota, differences in the net rate of absorption from the gut for the individual catabolites, and differences in subsequent mammalian metabolism and inherent potency.

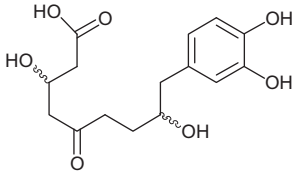

Figure 9. 9-(3′,4′-dihydroxy phenyl)-3,8-dihydroxy-5-keto-nonanoic acid.

4.4 Route of catabolism

Although several catabolites have been identified, these may be linked by more than one route of catabolism and from the present data it is not possible for either (–)-epicatechin (**1**) or procyanidin B2 (**2**) to define precisely the substrate flux following any particular route. For **1**, a plausible first step is reductive cleavage of the C-ring yielding **3** followed by cleavage of the A-ring as suggested for *Eubacterium oxidoreducens* catabolism of phloroglucinol [35] yielding by analogy 9-(3′,4′-dihydroxyphenyl)-3,8-dihydroxy-5-keto-nonanoic acid (Fig. 9). Progressive shortening of the aliphatic chain by α- and β-oxidations and lactonisation would generate the observed C_6–C_5, C_6–C_3, C_6–C_2 and C_6–C_1 skeletons and repeated dehydroxylations, favouring C4′ and the aliphatic side chain, would explain the remaining catabolites.

The results of this study indicate that early cleavage of the C4→C8 interflavan bond can occur but that it accounts for less than 10% of procyanidin B2 (**2**), although it is clear that this route may assume greater importance depending on the competence of the flora [34]. The observation also of five unique catabolites that retain the interflavan bond suggests that the early catabolism of **2** is likely to be similar to that of

1 ((–)-epicatechin). Although in theory there are two potential sites for reductive cleavage of the C-ring, it is likely that the site in the ''upper'' unit will be more accessible to the enzyme.

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The authors have declared no conflict of interest.

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