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Metabolism of green tea catechins by the human small intestine

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Abstract

Numerous studies have shown that green tea polyphenols can be degraded in the colon, and there is abundant knowledge about the metabolites of these substances that appear in urine and plasma after green tea ingestion. However, there is very little information on the extent and nature of intestinal degradation of green tea catechins in humans. Therefore, the aim of this study presented here was to examine in detail the microbial metabolism and chemical stability of these polyphenols in the small intestine using a well-established *ex vivo* model. For this purpose, fresh ileostomy fluids from two probands were incubated for 24 h under anaerobic conditions with (+)-catechin (C), (-)-epicatechin (EC), (-)-epicatechin 3-*O*-gallate (ECG), (-)-epigallocatechin (EGC), (-)-epigallocatechin 3-*O*-gallate (EGCG) and gallic acid (GA). After lyophilization and extraction, metabolites were separated, identified and quantified by high performance liquid chromatography-photodiode array detection (HPLC-DAD) and HPLC-DAD-tandem mass spectrometry. Two metabolites of EC and C -- 3',4',5'-trihydroxyphenyl- γ -valerolactone and 3',4'-dihydroxyphenyl- γ -valerolactone -- were identified. In addition, 3',4',5'-trihydroxyphenyl- γ -valerolactone was detected as a metabolite of EGC, and (after 24 h incubation) pyrogallol as a degradation product of GA. Cleavage of the gallic acid esters of EGCG and ECG was also observed, with variations dependent on the sources (probands) of the ileal fluids, which differed substantially microbiotically. The results provide new information about the degradation of green tea catechins in the gastrointestinal tract, notably that microbiota-dependent liberation of gallic acid esters may occur before these compounds reach the colon.

Key words green tea, small intestine, metabolism, catechins, gallic acid

Abbreviations

C	(+)-catechin
EC	(-)-epicatechin
ECG	(-)-epicatechin 3- <i>O</i> -gallate
EGC	(-)-epigallocatechin
EGCG	(-)-epigallocatechin 3- <i>O</i> -gallate
GA	gallic acid
HPLC-DAD	high performance liquid chromatography-photodiode array detection
MS/MS	tandem mass spectrometry
IS	internal standard
DMSO	dimethylsulfoxide

1 Introduction

Tea is the second most frequently consumed beverage globally, with a consumption of approximately 43.8 L per year [1, 2]. It is prepared from the dried leaves of *Camellia sinensis* in non-fermented (green tea), semi-fermented (oolong tea) or fermented (black tea) forms.

Green teas, which account for ~20% of global consumption [3], have well-established beneficial health effects, which are attributed to their polyphenolic contents, particularly contents of a subclass of flavanols the catechins [1]. Catechins are present in higher quantities in green tea than in other types of tea since they are degraded in the manufacturing processes involved in the production of black and oolong teas [4].

In recent decades there has been intense interest in the beneficial health effects of green tea polyphenols. A number of studies have shown them to have antioxidant [5], antibacterial [6] and anti-inflammatory [7] activities when ingested in the human diet. The consumption of green tea has also been associated with reduced risks of cancer [3, 8-11] and cardiovascular disease [12]. Furthermore, flavanols are known to effectively lower plasma low density lipoprotein levels [13]. These effects have been attributed mostly to the polyphenolic compounds of tea, namely catechins, which represent about 70% of polyphenolic content of green tea [14]. Structures of five major isolated catechins from different green teas are shown in figure 1, including: (+)-catechin (C), (-)-epicatechin (EC), (-)-epicatechin 3-*O*-gallate (ECG), (-)-epigallocatechin (EGC), (-)-epigallocatechin 3-*O*-gallate (EGCG) and gallic acid (GA) [15].

Due to their array of biological properties tea polyphenols, particularly catechins, have attracted the interest of various nutritional researchers [16, 17]. To understand the processes mediating the beneficial health effects of green tea catechins *in vivo* it is essential to acquire information on their bioavailability, absorption and metabolism. Therefore, a number of studies have examined and described various aspects of the biotransformation of tea flavanols

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3 by human, mice and rat gut microflora [18]. For example, [19] detected the formation of two
4
5 valerolactones after ingestion of radiolabelled compounds in rats and guinea pigs.
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7 Valerolactones have also been identified as metabolites of catechins in human urine and
8
9 plasma after oral ingestion of cups of green tea (1.2 g green tea powder in 300 ml hot water)
10
11 [20, 21]. In addition, [22] observed these valerolactone metabolites after *in vitro* incubation of
12
13 EGCG, ECG, EC and EGC for 24 h with a human fecal suspension. Other studies have
14
15 demonstrated glucuronidation, sulfation and methylation of catechins in the liver, kidney,
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17 brain and gastrointestinal tract of rats and mice [18, 23-25]. Further, in a recently published
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19 study, more than 20 metabolites -- glucuronides, methylated and ring-fission products -- have
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21 been detected in human urine after ingestion of a green tea solution using HPLC-ESI-MS/MS
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23 [26]. In addition, [27] have investigated food matrix effects on the absorption and metabolism
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25 of catechins using samples from five ileostomy volunteers who had consumed a green tea
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27 extract. The most abundant metabolites found in the urine of these subjects were sulfates of
28
29 catechin, epicatechin and their methylated forms. After ingestion of 200 mg green tea extract
30
31 43% of the ingested flavan-3-ols were recovered in ileal fluid. In another *ex vivo* study, tea
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33 catechins have been shown to be unstable in intestinal juice and buffered systems with pH
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35 >7.4, notably the concentration of catechins incubated in Krebs-Ringer buffer decreased by
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37 80% within an hour [28, 29].
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46 The specific objective of the study presented here was to examine in detail the microbial
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48 metabolism and chemical stability of green tea-derived catechins (shown in figure 1) in the
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50 small intestine under experimental conditions. More specifically, tea flavan-3-ols were
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52 incubated with fresh ileostomy fluid (obtained from volunteers with terminal ileostomies) and
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54 the metabolites formed were identified and quantified using HPLC-DAD and HPLC-ESI-
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56 MS/MS. In addition, the microbiota and enzymatic activities of the ileostomy effluents were
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58 characterized.
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2 Materials and Methods

2.1 Subjects

Two females between 45 and 60 years old, both with a terminal ileostomy, donated ileostomy effluents voluntarily for this study. Both probands suffered from Crohn's disease, without any involvement of the ileum and had undergone colectomy (with no surgical ileal resection) several years prior to the study, but were otherwise healthy.

A leaflet was given to both probands, asking them to avoid consuming food such as tea containing EC, EGC, EGCG, ECG, C and GA for 48 h before their ileostomy bag was removed. No drugs such as antibiotics were administered. The study protocol was approved by the Ethics Committee of the Mainz Medical Association (Landesärztekammer, no. 837.242.07 (5777)) in 2007.

2.2 Chemicals

All chemicals and solvents were of analytical grade. The flavanols (+)-catechin (C), (-)-epicatechin (EC), (-)-epigallocatechin (EGC), (-)-epigallocatechin 3-*O*-gallate (EGCG) and (-)-epicatechin 3-*O*-gallate (ECG) were purchased from Extrasynthèse (Lyon, France), while gallic acid (GA) and *p*-nitrophenol 3-*O*- α -L-rhamnopyranoside (*p*-nprha) were ordered from Sigma Aldrich (Munich, Germany). The reference substance 1,2,3-trihydroxybenzol (pyrogallol) and internal standard (IS) 3,4,5-trimethoxycinnamic acid were also purchased from Sigma Aldrich (Munich, Germany), while 3',4',5'-trihydroxyphenyl- γ -valerolactone and 3',4',-dihydroxyphenyl- γ -valerolactone were kindly provided by Prof. Rice (State University of New Jersey, USA).

2.3 Preparation of ileal fluids

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3 The two healthy volunteers (A and B) removed their fresh ileostomy bags, which were
4 immediately closed and placed in an anaerobic box containing Anaerocult[®] (Merck,
5 Darmstadt, Germany) to provide an anaerobic atmosphere. The samples were transported to
6 the laboratory at +4°C and the anaerobic box was placed in an anaerobic chamber flushed
7 with an 80:20 N₂:CO₂ Aligal gas mixture (Air Liquide, Kaiserslautern, Germany). The ileal
8 fluids from volunteers A and B were each diluted with an equal volume of anaerobic
9 carbonate-phosphate buffer, following [30], and filtered through a strainer to remove
10 particles. The resulting filtrates were designated ileal preparations A and B, respectively, and
11 used for the incubation experiments (see below).
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27 **2.4 Incubation conditions**

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29 Portions (10 µl) of 25 mM stock solutions of the test flavan-3-ols, GA and *p*-nitrophenol 3-*O*-
30 α -L-rhamnopyranoside in dimethylsulfoxide (DMSO) were placed in separate, small
31 incubation tubes (Sarstedt, Nuembrecht, Germany) together with 2.5 ml portions of ileal
32 samples A and B. The tubes were closed airtight, shaken and incubated for 0, ½, 1, 2, 4, 6, 8
33 and 24 h at 37 °C. The microbial reactions were then stopped by placing the tubes in liquid
34 nitrogen. As controls, samples with carrier (10 µl DMSO) and human inoculum but no test
35 substance, and others with test substances (C, EC, ECG, EGC, EGCG and G) but no inoculum
36 (stability controls) were also prepared and treated in the same manner. In addition, the
37 stability of each substance in carbonate-phosphate buffer without inoculum under anaerobic
38 conditions was evaluated following the above protocol. Each incubation experiment was
39 performed in triplicate.
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58 **2.5 Sample preparation**

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3 Frozen samples were lyophilized and extracted twice in 1 ml portions of a 70:30 (v/v)
4 methanol:water mixture containing 1% acetic acid, with 10-min treatments in an ultrasonic
5 bath. The two extracts from each sample were then pooled, centrifuged for 10 min at 5.000
6 rpm and filtered using 0.45 μm membrane filters (Buddeberg GmbH, Mannheim, Germany).
7
8 Finally, the IS 3,4,5-trimethoxycinnamic acid was added and 200 μl portions of each pooled
9 extract were analyzed by HPLC-DAD and HPLC-ESI-MS/MS (see below for
10 chromatographic conditions). The recovery rates of the catechins ranged from 39 to 99%.
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22 **2.6 HPLC-DAD data analysis**

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24 All samples were analysed using a Hewlett-Packard 1090 HPLC station equipped with a
25 photodiode array detector (DAD) (Waldbronn, Germany) and a 4.6 x 250 mm, 5 μm particle
26 size Symmetry C₁₈ column (Waters, Eschborn, Germany). The column oven temperature was
27 adjusted to 40°C, and the mobile phase consisted of aqueous 0.1% (v/v) formic acid (A) and
28 acetonitrile (B), in a 40-min linear gradient of 1-50% (B) at a flow rate of 1 ml/min. In each
29 case the injection volume was 20 μl . Eluting substances were identified by comparing their
30 retention times and UV spectra with those of authentic references, and the acquired data were
31 evaluated with Hewlett-Packard Chemstation[®] Software. To quantify the compounds
32 calibration curves (at appropriate wavelengths according to the absorption maxima of the
33 compounds) were generated. Linear responses were obtained for all the compounds at
34 concentrations up to 1 g/L, and their limits of quantification (LOQ) and determination (LOD),
35 defined as signal to noise ratios of 3:1 and 10:1, ranged from 0.6 to 4.0 mg/L and 0.1 to 0.6
36 mg/L, respectively.
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55 To establish degradation rates for each substance, in $\mu\text{mol/h}$, a tangent was drawn through the
56 inflection points of the degradation curves obtained from analyses of samples incubated for
57 various times, as described above, and a linear equation was fitted to it [31].
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2.7 HPLC-ESI-MS/MS data analysis

To identify flavan-3-ols and their corresponding metabolites a HPLC-ESI-MS/MS system with a SCIEX API 3200 MS/MS spectrometer equipped with an ESI interface (Applied Biosystems, Darmstadt, Germany), and a Jasco PU-980 pump (Groß-Umstadt, Germany) was used. The chromatographic system, including the column, mobile phase gradient, flow rate and injection volume, were all the same as in the HPLC-DAD analyses described above. MS analyses of eluting substances were carried out in negative ion, full-scan (100 to 900 atomic mass units) mode with a total scan duration of 1.0 s. Following optimization of the ionization, by infusing (-)-epicatechin with a syringe pump, the capillary temperature was set at 450 °C and the spray capillary voltage at -4.5 kV. Nitrogen was used as curtain gas (25 psi), nebulizer gas (GS1, 50 psi) and turbo gas (GS2, 40 psi). The declustering potential was -40 V and the entrance potential -4.5 V. The collision cell was operated with nitrogen, and a collision energy of -30 V was used. Both quadrupoles were set at unit resolution, and data were acquired and evaluated by Analyst Software 1.4.2 (Applied Biosystems, Darmstadt, Germany). The quasimolecular ions and product ion spectra obtained were compared to those of previously analyzed standards.

2.8 Api ZYM Tests

Api ZYM Test strips (Biomérieux, Lyon, France) were inoculated and incubated according to the manufacturer's instructions. Briefly, 2 ml of fresh ileal fluid from each proband was mixed with 4 ml distilled water, centrifuged at 2.000 rpm for 2 min, then each microcup of a test strip was inoculated with 65 µl of the resulting supernatant. The test strips were closed and incubated for 4 h under anaerobic conditions at 37 °C. After the incubation one drop of each of the two API reagents A and B were added to each microcup. Within 5 min colour

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3 reactions occurred and the colour intensity could be evaluated using an Api ZYM colour
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5 reaction chart.
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10 **2.9 Microorganism Diagnostics**

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12 In order to characterise the microbiota in the ileal fluids we sent cooled (+4°C) ileostomy
13 samples from each volunteer to an accredited laboratory (L+S AG, Bad Bocklet, Germany) to
14 determine the abundance of specific classes of microorganisms in them using standard aerobic
15 and anaerobic cultivation techniques.
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25 **3 Results**

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27 In order to imitate the anaerobic metabolism of the flavan-3-ols (C, EC, ECG, EGC, EGCG)
28 and GA in the upper gastrointestinal tract, an *ex vivo* model simulating passage through the
29 small intestine was used, in which the studied compounds were incubated for up to 24 h under
30 anaerobic conditions with inocula from the ileostomists. Samples were drawn at 30 min, 1, 2,
31 4, 6, 8, and 24 h then extracted and analyzed by HPLC-DAD to quantify the parent
32 compounds and metabolites, and by HPLC-ESI-MS/MS to confirm their identities.
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44 **3.1 Stability of the compounds in the carbonate-phosphate buffer**

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46 The stability of each studied compound in the absence of ileostomy fluid was evaluated by
47 incubating them in anaerobic carbonate-phosphate buffer solution for 24 h and analyzing the
48 solution at various time points. No degradation of C, EC, ECG, EGC, EGCG or GA was
49 observed within the first 8 h of these incubations (data not shown).
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59 **3.2 Recoveries of the flavanols from lyophilized inoculum**

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3 To determine the flavanol recovery rates provided by the sample preparation procedure,
4 lyophilized inocula from probands A and B were spiked with 25 mM of the studied
5 compounds, subjected to the procedure and then analyzed in the same manner as the samples.
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10 The recoveries of the incubated substances are shown in table 1.
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13 **3.3 Degradation of green tea catechins in human ileostomy fluid**

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18 Degradation rates of the green tea catechins (summarised in table 2) were consistently lower
19 when incubated with the ileostomy preparations from proband A than with those from
20 proband B. The fates of the specific catechins are described in more detail in the following
21 sections.
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26
27 *ECG*. The kinetics of the degradation of ECG and the appearance of metabolites observed
28 during incubations of the substance with fresh human ileostomy preparations for 24 h are
29 shown in figure 2. During the first two hours ECG was almost completely degraded to GA
30 and EC, at 0.123 $\mu\text{mol/h}$, in the presence of the ileal preparation from proband B. After
31 reaching peak concentrations at two hours the level of GA then decreased, while the level of
32 EC remained roughly constant. In the presence of ileal preparation A the degradation rate of
33 ECG was much slower (0.024 $\mu\text{mol/h}$), and a substantial proportion (33%) still remained after
34 the 24 h incubation. Nevertheless, GA and EC were still detected within 2 h, and their levels
35 rose for ca. 4 h then remained roughly constant.
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49 Comparison of retention times and the UV spectra of the products with those of authentic
50 references confirmed their identities. The expected quasimolecular ions of m/z 169 for GA
51 and m/z 289 for EC were observed by HPLC-ESI-MS in negative ion mode, and identical
52 profiles of daughter ions to those obtained from commercially available references
53 (characteristic fragments with m/z ratios of 69, 79, 97, 107 and 125 for GA, and m/z ratios of
54 109, 125, 137, 151 and 203 for EC). After a further 6 h incubation traces of the metabolites
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3 3',4',5'-trihydroxyphenyl- γ -valerolactone (m/z 223), 3',4',-dihydroxyphenyl- γ -valerolactone
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5 (m/z 207), and pyrogallol (m/z 125) were detected by HPLC-ESI-MS/MS, and confirmed by
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7 comparison of retention time and spectra of authentic references.
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10 Figure 3 shows two HPLC chromatograms obtained from samples of the incubations of ECG
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12 (which eluted after 20.5 min, indicated by the numeral 1) with ileal preparation B collected at
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14 the start of the incubation (a) and after 2 h (b). The results clearly show that the metabolites
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16 GA (3, 7.1 min) and EC (4, 16.3 min) were formed during the incubation with the ileostomy
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18 fluid B.
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22 The kinetics of the degradation of EGCG and the appearance of metabolites observed during
23
24 incubations of the substance with fresh human ileostomy preparations for 24 h are shown in
25
26 figure 4. The degradation patterns were very similar to those observed for EGC, in that the
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28 substance was degraded more rapidly in contact with ileal preparation B than with ileal
29
30 preparation A, but in both cases two metabolites were detected within 2 h. Comparison of
31
32 their UV spectra, retention times and mass spectra in negative ionisation mode with those of
33
34 authentic standards confirmed these metabolites to be GA and EGC (quasimolecular ions m/z
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36 169 and m/z 305, respectively. The structures of the degradation products are shown in figure
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41 1).
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43 In the presence of ileal preparations from both probands the level of the degradation product
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45 EGC rapidly rose in the first 2 h. However, with preparation A it subsequently declined, while
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47 in the presence of preparation B it remained roughly constant for the following 22 h. In
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49 addition, GA levels peaked after 4 h then gradually declined during the following 20 h in the
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51 presence of preparation B, while they rose to a much lower maximum after 2 h with ileal
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53 preparation A then remained roughly constant. After 8 h incubation traces of the degradation
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55 products of EGC pyrogallol and 3',4',5'-trihydroxyphenyl- γ -valerolactone were detectable by
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57 HPLC-ESI-MS/MS (data not shown).
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3 *EGC*. The degradation of EGC in the presence of preparations from both probands was quite
4 similar, as shown in figure 5. Only slow degradation (0.026 and 0.033 $\mu\text{mol/h}$ with
5 preparations A and B, respectively) was observed within the incubation period. Traces of the
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metabolite 3',4',5'-trihydroxyphenyl- γ -valerolactone (m/z 223) were identified, by comparing
its HPLC-ESI-MS/MS product ion spectra (dominated by fragments with m/z ratios of 124,
138, and 179) with that of an authentic reference. Due to the low concentration of the
metabolite it could not be quantified.

C and EC. As shown by the illustrative time courses of changes in the levels of C presented in
figure 6, this substance was very slowly degraded (at 0.27 $\mu\text{mol/h}$) in the presence of the ileal
preparation from proband A, and most of the initial amount remained after 24 h incubation.
With preparation B the concentration declined much more rapidly in the first 2 h (at 0.051
 $\mu\text{mol/h}$), but the level remained almost stable thereafter. The incubations with EC yielded
similar patterns. In addition, 3',4',5'-trihydroxyphenyl- γ -valerolactone and 3',4',-
dihydroxyphenyl- γ -valerolactone were detected as metabolites of both EC and C. The
identities of these metabolites were confirmed by HPLC-ESI-MS analysis (quasimolecular
ions m/z 223 and 207, respectively, with identical product ion profiles to those of authentic
standards). C was also detected as an EC metabolite. Due to the low concentrations of the
metabolites (less than 1% of the initial concentration of the parent compounds) they could not
be quantified.

GA. GA was degraded at similar rates by the preparations from probands A and B (0.022 and
0.051 $\mu\text{mol/h}$, respectively), continuously during the 24 h incubations (figure 7), and at the
end of the incubations approximately 12% of the initial concentrations were still detectable in
both cases. However, we would specify the time periods for which the rates apply, because it
would be impossible for the final concentrations to be so similar if the rates diverged so much

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3 throughout the incubations. The metabolite pyrogallol was identified in the samples by
4 comparing its retention time and UV spectra with those of an authentic reference, and verified
5 by HPLC-ESI-MS/MS in negative ionisation mode, in which its retention time and product
6 ion spectrum (dominant fragments with m/z ratios of 69, 79, 97 and 125) was identical to that
7 of the standard.
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18 **3.4 Glycosidase activity of the ileal microflora**

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20 The glycosidase activity of the microflora in the ileostomy fluids was assayed by incubating
21 *p*-nitrophenol 3-*O*- α -L-rhamnopyranoside (*p*-nprha) under anaerobic conditions with the
22 ileostomy fluids, as described above, and monitoring its degradation. The results showed that
23 the ileostomy fluids differed significantly in glycosidase activity; complete liberation of *p*-
24 nitrophenol from *p*-nprha took 0.5 h and 1 h in the presence of the preparations from probands
25 A and B, respectively.
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36 **3.5 Analyses of the ileal fluids**

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38 Since the pH is also an important factor for the stability of catechins in the ileostomy fluid, it
39 was determined before all experiments and was found to be consistently between 6.08 and
40 6.24. In addition, characterisation by a commercial laboratory (L+S AG, Bad Bocklet,
41 Germany) showed there were significant differences in the microbial flora of the ileal fluids
42 of the two probands in terms of concentrations of fungi and bacteria (anaerobic and aerobic)
43 (see table 3). However, both preparations were able to cleave 2-naphthyl caprylate, according
44 to the Api Zym tests. Heating of fresh ileostomy fluid to inactivate the intestinal microflora
45 significantly reduced the degradation of the compounds under study.
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4 Discussion

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3 In the presented work we used an *ex vivo* ileostomy model to study the microbial metabolism
4 of green tea catechins and gallic acid in the small intestine. The catechins and their
5 metabolites were quantified by HPLC-DAD and identified by HPLC-ESI-MS/MS.
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10 All of the studied tea catechins (C, EC, ECG, EGC, EGCG) and GA were metabolised during
11 incubation with human ileal fluid, and cleavage of the esters ECG and EGCG into the
12 corresponding aglycones and GA was observed. Furthermore, pyrogallol was identified as a
13 degradation product of GA, and two metabolites of C, EC and EGC were identified by HPLC-
14 ESI-MS/MS (3',4',5'-trihydroxyphenyl- γ -valerolactone and 3',4'-dihydroxyphenyl- γ -
15 valerolactone). There is no evidence of valerolactone metabolites in black tea after the
16 fermentation process. The fermentation is performed by a polyphenol oxidase catalysed
17 oxidation of fresh tea leaf. During this process, polyphenols such as catechins are oxidized
18 which is associated with an increase in monoterpene alcohol [32]. Due to this fermentation
19 process 60 to 80% of green tea catechins are transformed into theaflavins, thearubigins and
20 other epimerization products as reported by literature [33, 34].
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36 Despite our *ex vivo* findings of catechin degrading activities and observations of cleavage of
37 the gallate moieties of EGCG and ECG in ileal fluids, in a study where five volunteer
38 ileostomists ingested 300 ml portions of a solution of a green tea extract (Polyphenon E) [27]
39 found that some catechins, mainly EGCG, reach the colon of healthy humans. In addition,
40 cleavage of the ester bonds of galloylated catechins by intestinal microbiota has been
41 observed in incubations with the caecum of freshly slaughtered pigs [35]. However, [36]
42 found that 31% of ingested green tea flavanols were absorbed in the small intestine and
43 preceding parts of the gut in the 24 h following ingestion. Furthermore, a microbial esterase
44 activity has been detected in human saliva [37] and observed in our assays by cleavage of the
45 substrate 2-naphthyl caprylate in the Api ZYM tests. Therefore, microbial esterase activity
46 was presumably responsible for the cleavage of ECG and EGCG during our incubations,
47 which were intended to simulate passage through the small intestine. Overall, the findings of
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3 this and several previous studies indicate that some catechin metabolism (at least) occurs
4 during passage through this part of the gut.
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8 However, extensive bacterial degradation of catechins may also occur in the colon. Meselhy
9 and coworkers [22] incubated tea catechins with a human fecal suspension, and most of the
10 incubated catechins (EGCG, ECG, EC, EGC) were metabolized to 3',4',5'-trihydroxyphenyl-
11 γ -valerolactone, 3',4'-dihydroxyphenyl- γ -valerolactone, 3'-hydroxyphenyl- γ -valerolactone
12 and other metabolites. Two of these substances (3',4',5'-trihydroxyphenyl- γ -valerolactone
13 and 3',4'-dihydroxyphenyl- γ -valerolactone) have also been detected in our studies and by
14 other groups in human urine and blood after tea ingestion [20, 21, 38]. These metabolites have
15 also been observed (presumably due to microbial metabolism) following oral administration
16 of EC, EGC and GA in human plasma and urine, as well as rat and mouse tissues, by [39].
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18 The formation of GA and further degradation to pyrogallol in incubations of EGCG and ECG
19 with human fecal suspension have also been described [22]. However, our findings indicate
20 that some formation of GA and further metabolism to pyrogallol (at least) occurs by the end
21 of the small intestine since low amounts of pyrogallol were detected following our
22 incubations with ileal fluids. These divergences from previous findings may be due to the
23 rapid autoxidation of pyrogallol in the presence of oxygen during sample preparation [40]. All
24 metabolites we identified are shown in figure 8.
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28 Due to the substantial differences in the degradation rates observed in the incubations with
29 ileal preparations of probands A and B, the microbiotic flora of the ileal fluids were examined
30 (table 3), and substantial differences in their fungal and bacterial (anaerobic and aerobic)
31 populations were observed.
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35 Furthermore, glycosidase activity assays showed that the *p*-nprha degradation rate was much
36 higher in the inoculum from proband B than in the inoculum from proband A, providing
37 further indications of microbiological differences between them.
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3 The diversity of microbial populations in the human gastrointestinal tract has been previously
4 reported, and various groups have shown that diet and lifestyle can substantially affect human
5 microbiota, so there may be high intra- and inter-individual variations in intestinal bacteria
6 communities [41-43]. However, in contrast to previous authors, who found a catechin
7 metabolism in the colon, we observed (using our well-established *ex vivo* model) indications
8 that significant metabolism of green tea catechins may also occur in the upper part of the GIT
9 namely in the ileum. Further studies are needed to elucidate the metabolism and
10 bioavailability of catechins more comprehensively, in order to correlate their metabolism to
11 the disease-preventive effects of green tea.
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30 University of New Jersey, USA) for providing the valerolactone standards, and Dr. G.
31 Krammer/ Symrise GmbH & Co. KG (Holzminden, Germany) for their support with HPLC-
32 DAD equipment.
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5 References

- [1] Graham, H. N., Green tea composition, consumption, and polyphenol chemistry. *Prev. Med.* 1992, 21, 334-350.
- [2] McKay, D. L., Blumberg, J. B., The role of tea in human health: an update. *J. Am. Coll. Nutr.* 2002, 21, 1-13.
- [3] Ahmad, N., Feyes, D. K., Nieminen, A. L., Agarwal, R., Mukhtar, H., Green tea constituent epigallocatechin-3-gallate and induction of apoptosis and cell cycle arrest in human carcinoma cells. *J Natl Cancer Inst* 1997, 89, 1881-1886.
- [4] Lin, Y. S., Tsai, Y. J., Tsay, J. S., Lin, J. K., Factors affecting the levels of tea polyphenols and caffeine in tea leaves. *J. Agric. Food Chem.* 2003, 51, 1864-1873.
- [5] Shahrzad, S., Bitsch, I., Determination of gallic acid and its metabolites in human plasma and urine by high-performance liquid chromatography. *J. Chromatogr. B Biomed. Sci. Appl.* 1998, 705, 87-95.
- [6] Stapleton, P. D., Shah, S., Anderson, J. C., Hara, Y., *et al.*, Modulation of beta-lactam resistance in *Staphylococcus aureus* by catechins and gallates. *Int. J. Antimicrob. Agents* 2004, 23, 462-467.
- [7] Lambert, J. D., Rice, J. E., Hong, J., Hou, Z., Yang, C. S., Synthesis and biological activity of the tea catechin metabolites, M4 and M6 and their methoxy-derivatives. *Bioorg. Med. Chem. Lett.* 2005, 15, 873-876.
- [8] Lambert, J. D., Yang, C. S., Cancer chemopreventive activity and bioavailability of tea and tea polyphenols. *Mutat. Res.* 2003, 523-524, 201-208.
- [9] Katiyar, S. K., Mukhtar, H., Tea antioxidants in cancer chemoprevention. *J. Cell. Biochem. Suppl.* 1997, 27, 59-67.
- [10] Yang, C. S., Sang, S., Lambert, J. D., Hou, Z., *et al.*, Possible mechanisms of the cancer-preventive activities of green tea. *Mol. Nutr. Food Res.* 2006, 50, 170-175.
- [11] Zaveri, N. T., Green tea and its polyphenolic catechins: medicinal uses in cancer and noncancer applications. *Life Sci.* 2006, 78, 2073-2080.
- [12] Yamada, H., Watanabe, H., Tea polyphenols in preventing cardiovascular diseases. *Cardiovasc. Res.* 2007, 73, 439-440.
- [13] Miura, Y., Chiba, T., Miura, S., Tomita, I., *et al.*, Green tea polyphenols (flavan 3-ols) prevent oxidative modification of low density lipoproteins: an *ex vivo* study in humans. *J. Nutr. Biochem.* 2000, 11, 216-222.
- [14] Lakenbrink, C., Lapczynski, S., Maiwald, B., Engelhardt, U. H., Flavonoids and other polyphenols in consumer brews of tea and other caffeinated beverages. *J. Agric. Food Chem.* 2000, 48, 2848-2852.
- [15] Jin, Y., Jin, C. H., Row, K. H., Separation of catechin compounds from different teas. *Biotechnol. J.* 2006, 1, 209-213.
- [16] Scalbert, A., Manach, C., Morand, C., Remesy, C., Jimenez, L., Dietary polyphenols and the prevention of diseases. *Crit. Rev. Food Sci. Nutr.* 2005, 45, 287-306.
- [17] Yao, L. H., Jiang, Y. M., Shi, J., Tomas-Barberan, F. A., *et al.*, Flavonoids in food and their health benefits. *Plant Foods Hum. Nutr.* 2004, 59, 113-122.
- [18] Lambert, J. D., Lee, M. J., Lu, H., Meng, X., *et al.*, Epigallocatechin-3-gallate is absorbed but extensively glucuronidated following oral administration to mice. *J. Nutr.* 2003, 133, 4172-4177.
- [19] Das, N. P., Griffith, L., Studies on Flavonoid Metabolism - Metabolism of (+)-[14c]Catechin in Rat and Guinea Pig. *Biochem. J.* 1969, 115, 831-&.
- [20] Li, C., Meng, X., Winnik, B., Lee, M. J., *et al.*, Analysis of urinary metabolites of tea catechins by liquid chromatography/electrospray ionization mass spectrometry. *Chem. Res. Toxicol.* 2001, 14, 702-707.

- 1
2
3 [21] Li, C., Lee, M. J., Sheng, S., Meng, X., *et al.*, Structural identification of two metabolites
4 of catechins and their kinetics in human urine and blood after tea ingestion. *Chem. Res.*
5 *Toxicol.* 2000, *13*, 177-184.
6
7 [22] Meselhy, M. R., Nakamura, N., Hattori, M., Biotransformation of (-)-epicatechin 3-O-
8 gallate by human intestinal bacteria. *Chem. Res. Toxicol.* 1997, *45*, 888-893.
9 [23] Donovan, J. L., Crespy, V., Manach, C., Morand, C., *et al.*, Catechin is metabolized by
10 both the small intestine and liver of rats. *J. Nutr.* 2001, *131*, 1753-1757.
11 [24] Kuhnle, G., Spencer, J. P., Schroeter, H., Shenoy, B., *et al.*, Epicatechin and catechin are
12 O-methylated and glucuronidated in the small intestine. *Biochem. Biophys. Res. Commun.*
13 2000, *277*, 507-512.
14 [25] Tsang, C., Auger, C., Mullen, W., Bornet, A., *et al.*, The absorption, metabolism and
15 excretion of flavan-3-ols and procyanidins following the ingestion of a grape seed extract by
16 rats. *Br. J. Nutr.* 2005, *94*, 170-181.
17 [26] Sang, S., Lee, M. J., Yang, I., Buckley, B., Yang, C. S., Human urinary metabolite
18 profile of tea polyphenols analyzed by liquid chromatography/electrospray ionization tandem
19 mass spectrometry with data-dependent acquisition. *Rapid Commun. Mass Spectrom.* 2008,
20 *22*, 1567-1578.
21 [27] Auger, C., Mullen, W., Hara, Y., Crozier, A., Bioavailability of polyphenon E flavan-3-
22 ols in humans with an ileostomy. *J. Nutr.* 2008, *138*, 1535-1542.
23 [28] Zhu, Q. Y., Holt, R. R., Lazarus, S. A., Ensunsa, J. L., *et al.*, Stability of the flavan-3-ols
24 epicatechin and catechin and related dimeric procyanidins derived from cocoa. *J. Agric. Food*
25 *Chem.* 2002, *50*, 1700-1705.
26 [29] Zhu, Q. Y., Zhang, A., Tsang, D., Huang, Y., Chen, Z. Y., Stability of Green Tea
27 Catechins. *J. Agric. Food Chem.* 1997, *45*, 4624-4628.
28 [30] Lebet, V., Arrigoni, E., Amado, R., Measurement of fermentation products and substrate
29 disappearance during incubation of dietary fibre sources with human faecal flora. *Lebensm.*
30 *Wiss. Technol.* 1998, *31*, 473-479.
31 [31] Knaup, B., Kahle, K., Erk, T., Valotis, A., *et al.*, Human intestinal hydrolysis of phenol
32 glycosides - a study with quercetin and p-nitrophenol glycosides using ileostomy fluid. *Mol.*
33 *Nutr. Food Res.* 2007, *51*, 1423-1429.
34 [32] Wang, H., Provan, G. J., Helliwell, K., Tea flavonoids: their functions, utilisation and
35 analysis. *Trends Food Sci. Technol.* 2000, *11*, 152-160.
36 [33] Wiseman, S., Mulder, T., Rietveld, A., Tea flavonoids: Bioavailability in vivo and effects
37 on cell signaling pathways in vitro. *Antioxid. Redox Signal.* 2001, *3*, 1009-1021.
38 [34] Wang, Y., Ho, C. T., Polyphenolic Chemistry of Tea and Coffee: A Century of Progress.
39 *J. Agric. Food Chem.* 2009, *57*, 8109-8114.
40 [35] van't Slot, G., Humpf, H. U., Degradation and Metabolism of Catechin, Epigallocatechin-
41 3-gallate (EGCG), and Related Compounds by the Intestinal Microbiota in the Pig Cecum
42 Model. *J. Agric. Food Chem.* 2009, *57*, 8041-8048.
43 [36] Stalmach, A., Mullen, W., Steiling, H., Williamson, G., *et al.*, Absorption, metabolism,
44 and excretion of green tea flavan-3-ols in humans with an ileostomy. *Mol. Nutr. Food Res.*
45 2009, *54*, 323-334.
46 [37] Yang, C. S., Lee, M. J., Chen, L. S., Human salivary tea catechin levels and catechin
47 esterase activities: Implication in human cancer prevention studies. *Cancer Epidemiol.*
48 *Biomarkers Prev.* 1999, *8*, 83-89.
49 [38] Lee, M. J., Maliakal, P., Chen, L., Meng, X., *et al.*, Pharmacokinetics of tea catechins
50 after ingestion of green tea and (-)-epigallocatechin-3-gallate by humans: formation of
51 different metabolites and individual variability. *Cancer Epidemiol. Biomarkers Prev.* 2002,
52 *11*, 1025-1032.
53
54
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3 [39] Meng, X., Sang, S., Zhu, N., Lu, H., *et al.*, Identification and characterization of
4 methylated and ring-fission metabolites of tea catechins formed in humans, mice, and rats.
5 *Chem. Res. Toxicol.* 2002, *15*, 1042-1050.
6
7 [40] Marklund, S., Marklund, G., Involvement of Superoxide Anion Radical in Autoxidation
8 of Pyrogallol and a Convenient Assay for Superoxide-Dismutase. *Eur. J. Biochem.* 1974, *47*,
9 469-474.
10 [41] Jacobs, D. M., Deltimple, N., van Velzen, E., van Dorsten, F. A., *et al.*, H-1 NMR
11 metabolite profiling of feces as a tool to assess the impact of nutrition on the human
12 microbiome. *Nmr Biomed.* 2008, *21*, 615-626.
13 [42] Eckburg, P. B., Bik, E. M., Bernstein, C. N., Purdom, E., *et al.*, Diversity of the human
14 intestinal microbial flora. *Science* 2005, *308*, 1635-1638.
15 [43] Muyzer, G., Dewaal, E. C., Uitterlinden, A. G., Profiling of Complex Microbial-
16 Populations by Denaturing Gradient Gel-Electrophoresis Analysis of Polymerase Chain
17 Reaction-Amplified Genes-Coding for 16s Ribosomal-Rna. *Appl. Environ. Microbiol.* 1993,
18 59, 695-700.
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Figure legends

Fig. 1: Chemical structures of the most common catechins and gallic acid esters characterized in green tea.

Fig. 2: Changes in concentrations [μmol] of (-)-epicatechin 3-*O*-gallate (ECG) and its metabolites during incubations with preparations of human ileostomy fluid from two donors measured by HPLC-DAD at 280 nm. Proband A: ECG (■) and the degradation products GA (▲), EC (●). Proband B: ECG (□) and the degradation products GA (Δ), EC (○). Values are means of three determinations \pm SD.

Fig. 3: HPLC chromatograms recorded at 280 nm of samples obtained from anaerobic incubations of ECG with ileal preparation from proband B at 0 h (a) and after two hours of incubation (b). *, Ileostomy constituents: 1, ECG; 2, the internal standard 3,4,5-trimethoxycinnamic acid (IS); 3, GA; 4, EC. For experimental conditions see the materials and methods section.

Fig. 4: Changes in concentrations [μmol] of (-)-epigallocatechin 3-*O*-gallate and its metabolites during incubations with preparations of human ileostomy fluid from two donors measured by HPLC-DAD at 280 nm: proband A, EGCG (■) and the degradation products GA (▲), EGC (●); and proband B, EGCG (□) and the degradation products GA (Δ), EGC (○). Values are means of threefold determinations \pm SD.

Fig. 5: Changes in concentrations [μmol] of (-)-epigallocatechin (EGC) during incubations with preparations of human ileostomy fluid from two donors measured by HPLC-DAD at 280 nm: proband A (■) and proband B (□). Values are means of three determinations \pm SD.

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6 **Fig. 6:** Changes in concentrations [μmol] of (+)-catechin (C) during incubations with
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8 preparations of human ileostomy fluid from two donors measured by HPLC-DAD at 280 nm:
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10 proband A (■) and proband B (□). Values are means of three determinations \pm SD.
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15 **Fig. 7:** Changes in concentration [μmol] of gallic acid (GA) during incubations with
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17 preparations of human ileostomy fluid from two donors measured by HPLC-DAD at 280 nm:
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19 proband A (■) and B (□). Values are means of three determinations \pm SD.
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24 **Fig. 8:** Chemical structures of metabolites detected from the incubations of (+)-catechin, (-)-
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26 epicatechin and gallic acid with human ileostomy fluid.
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Tables

Tab. 1 Recovery rates for the sample preparation method used for the compounds under study
(n=3).

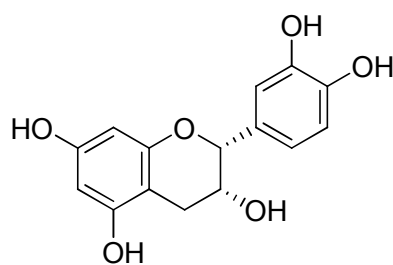
compound	recovery [%]
C	82 ± 13
EC	36 ± 2
ECG	74 ± 8
EGC	72 ± 9
EGCG	82 ± 5
GA	94 ± 5

Tab. 2 Degradation rates [$\mu\text{mol/h}$] of green tea catechins under study incubated with preparations of human ileostomy fluids from both probands

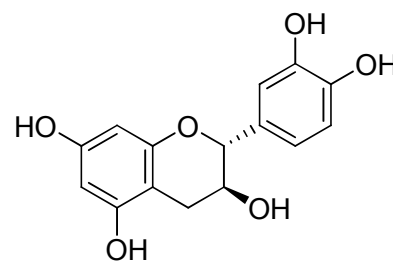
catechins	Degradation rate	Degradation rate
	proband A [$\mu\text{mol/h}$]	proband B [$\mu\text{mol/h}$]
C	0.027	0.051
EC	0.035	0.089
ECG	0.024	0.123
EGC	0.026	0.033
EGCG	0.015	0.113
GA	0.022	0.051

Tab. 3 Microbiota analysis. Significant differences between the microbiota of the two probands are marked bold. cfu = colony forming units. LOD = 10 [cfu/g]

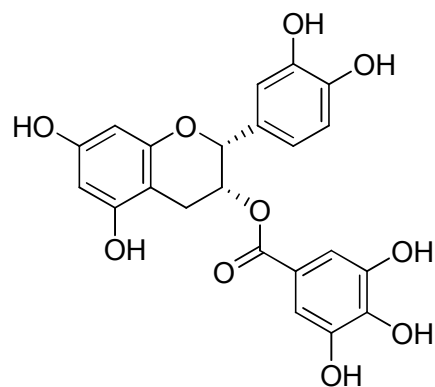
	proband A	proband B
	[cfu/g]	[cfu/g]
aerobian		
- <i>E.coli</i>	7*10⁷	2*10⁸
- <i>E.coli</i> -variants	< 10	< 10
- <i>Proteus sp.</i>	< 10	1*10⁸
- <i>Enterococcus sp.</i>	5*10⁶	< 10
- other aerob	< 10	< 10
anaerobian		
- <i>Bacteroides sp.</i>	3*10³	1*10⁶
- <i>Clostridium sp.</i>	< 10	< 10
- <i>Bifidobacterium sp.</i>	4*10²	3*10³
- <i>Lactobacilius sp.</i>	5*10²	3*10³
- other anaerobian	< 10	< 10
fungi		
- <i>Candida sp.</i>	< 10	< 10
- <i>Geotrichum sp.</i>	1*10⁴	< 10
- other fungi	< 10	< 10



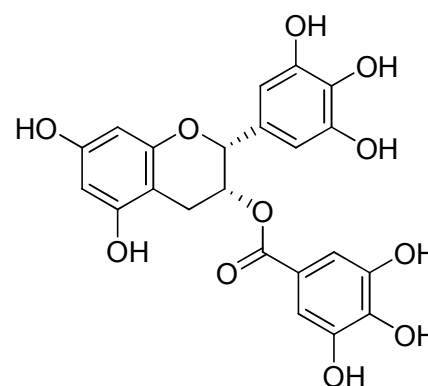
(-)-epicatechin (EC)



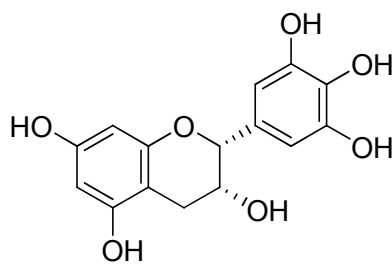
(+) -Catechin (C)



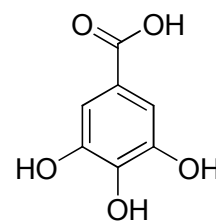
(-)-epicatechin 3-O-gallate (ECG)



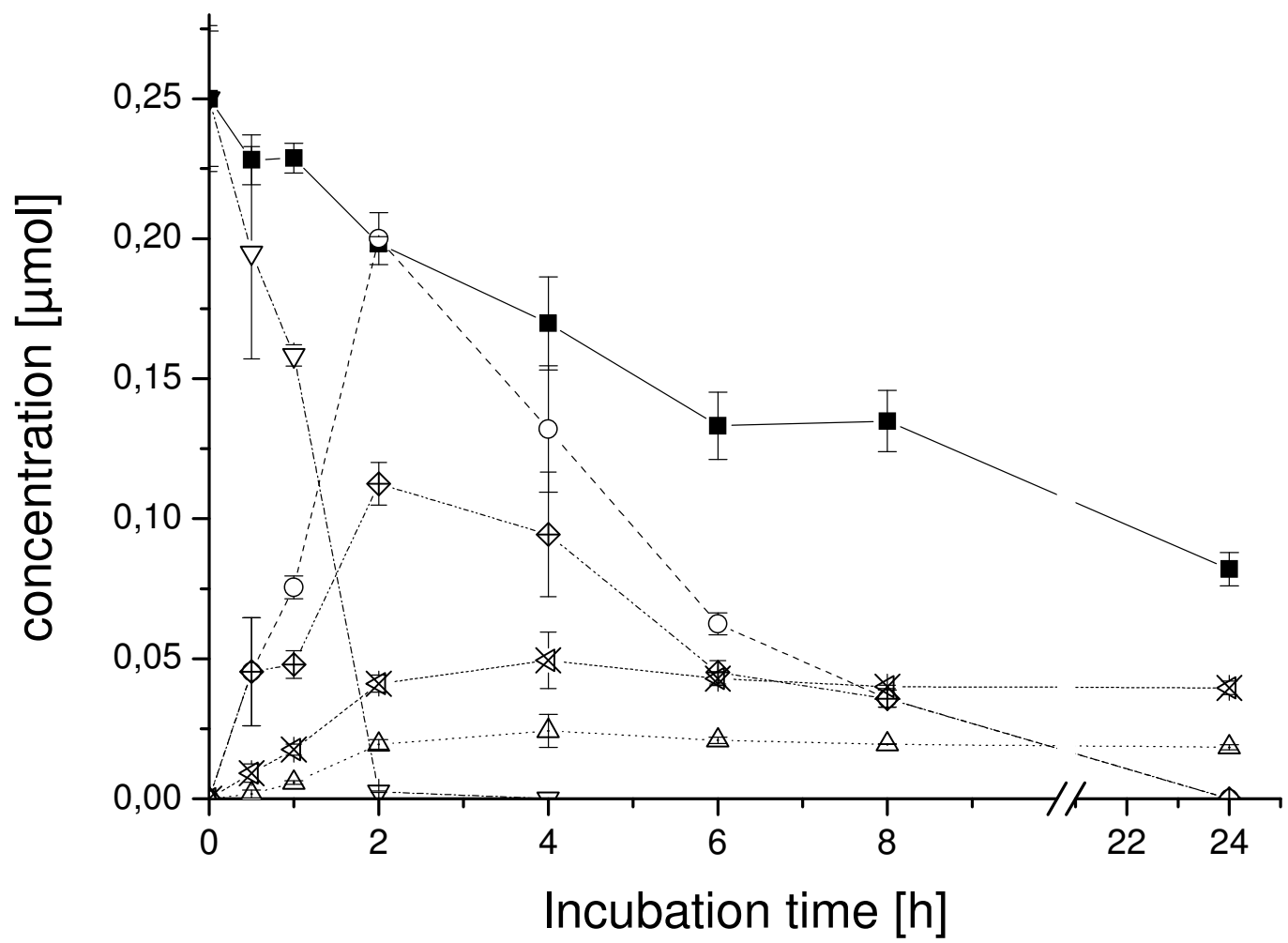
(-)-epigallocatechin 3-O-gallate (EGCG)

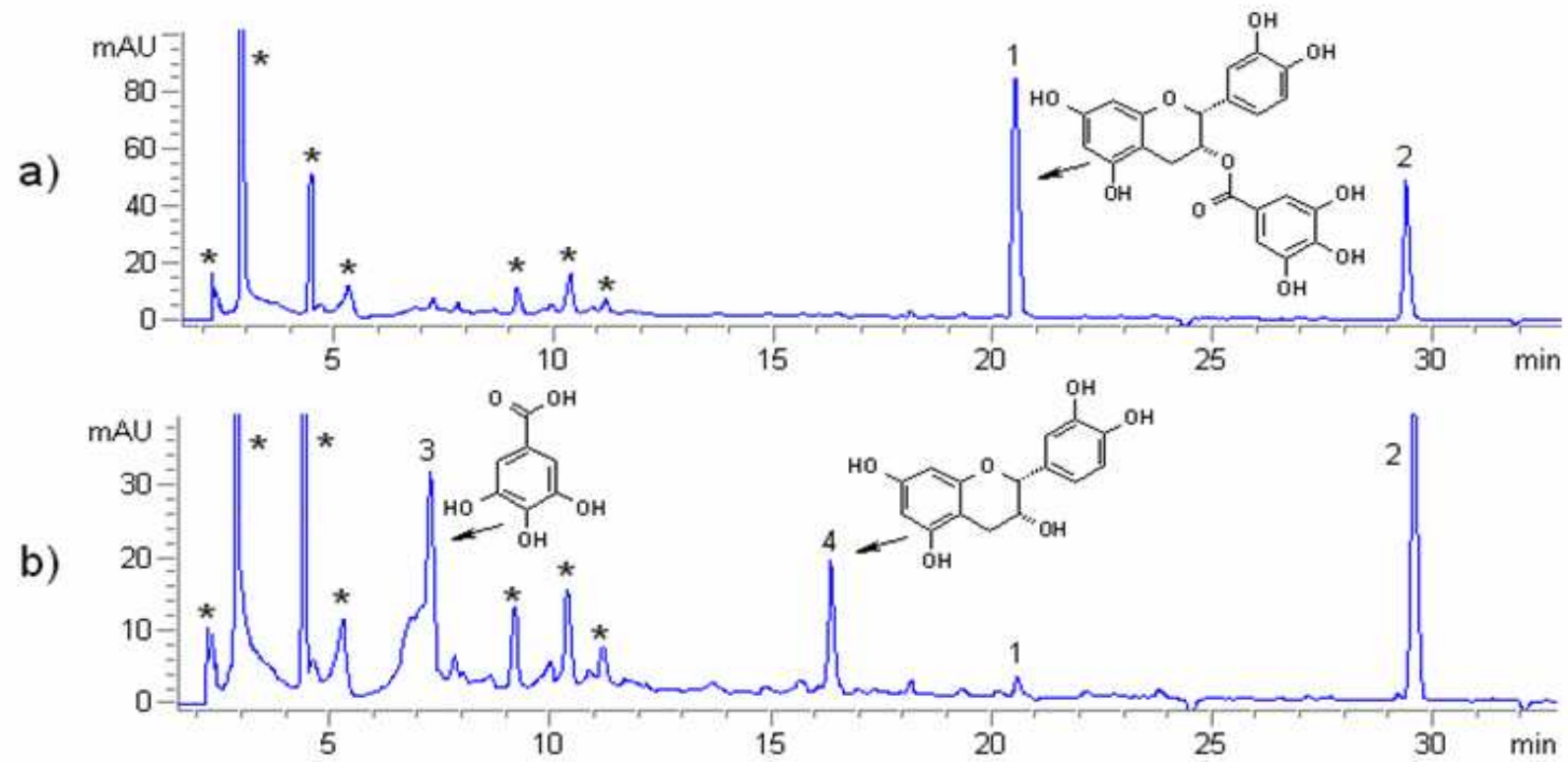


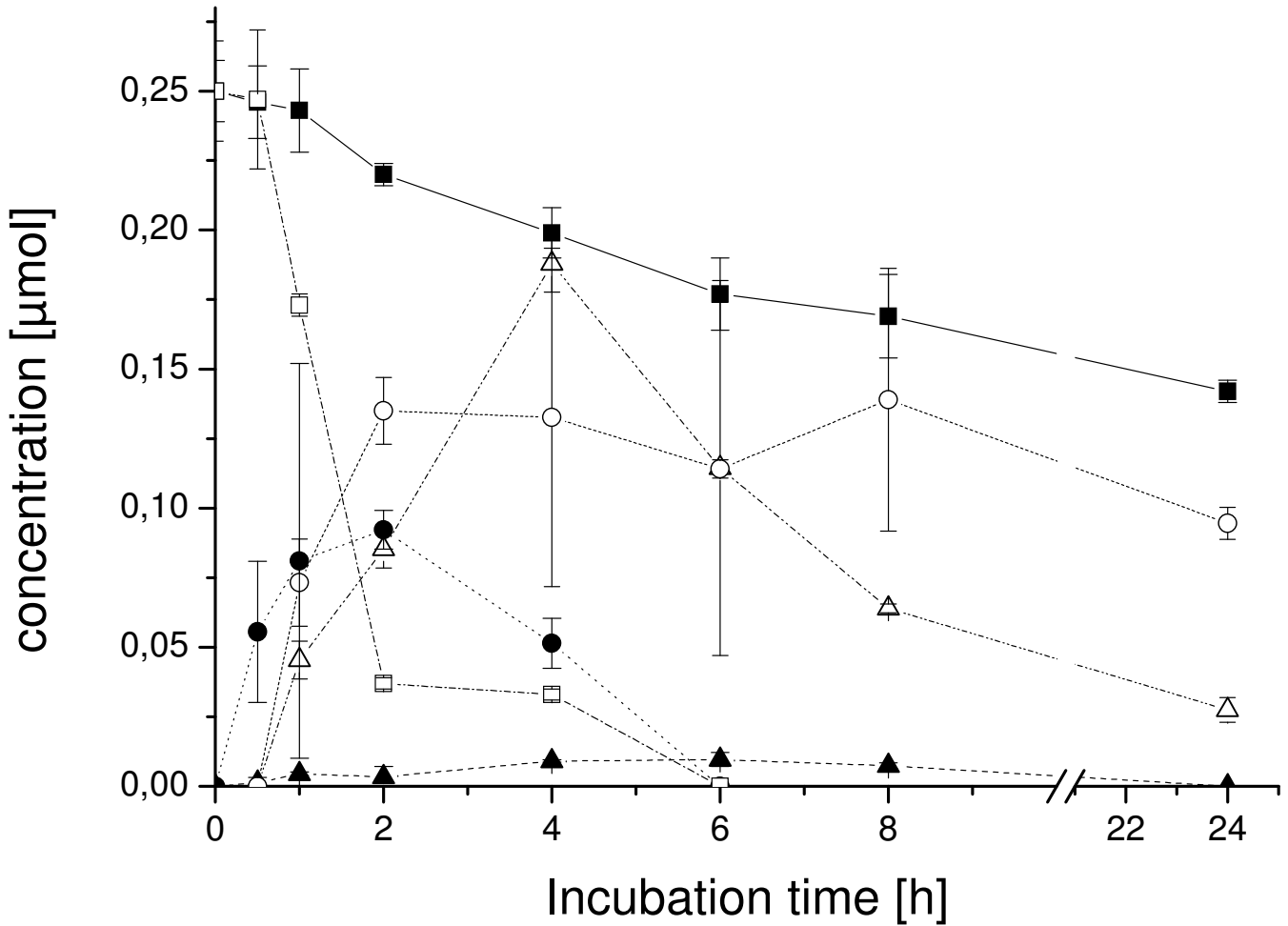
(-)-epigallocatechin (EGC)

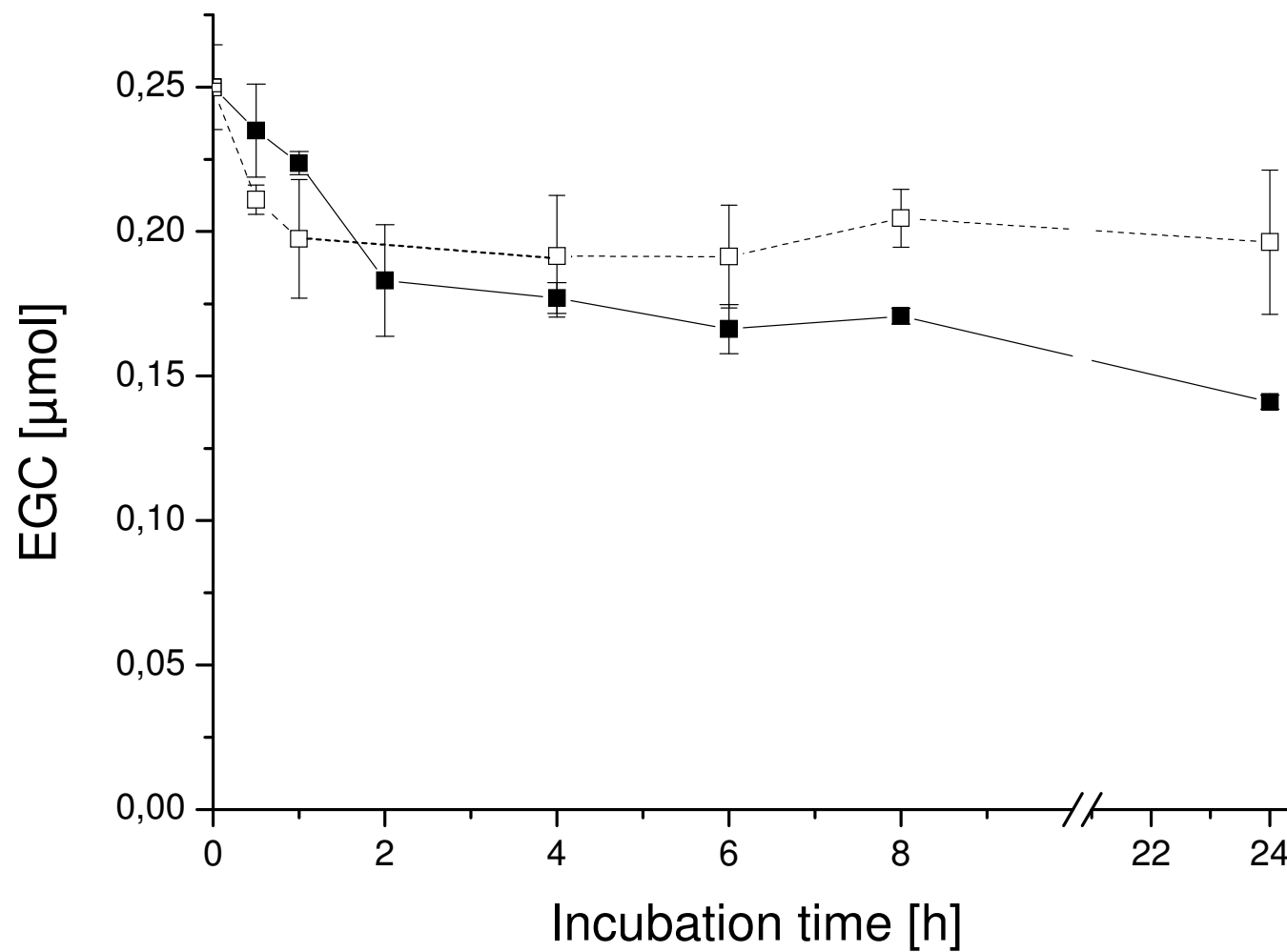


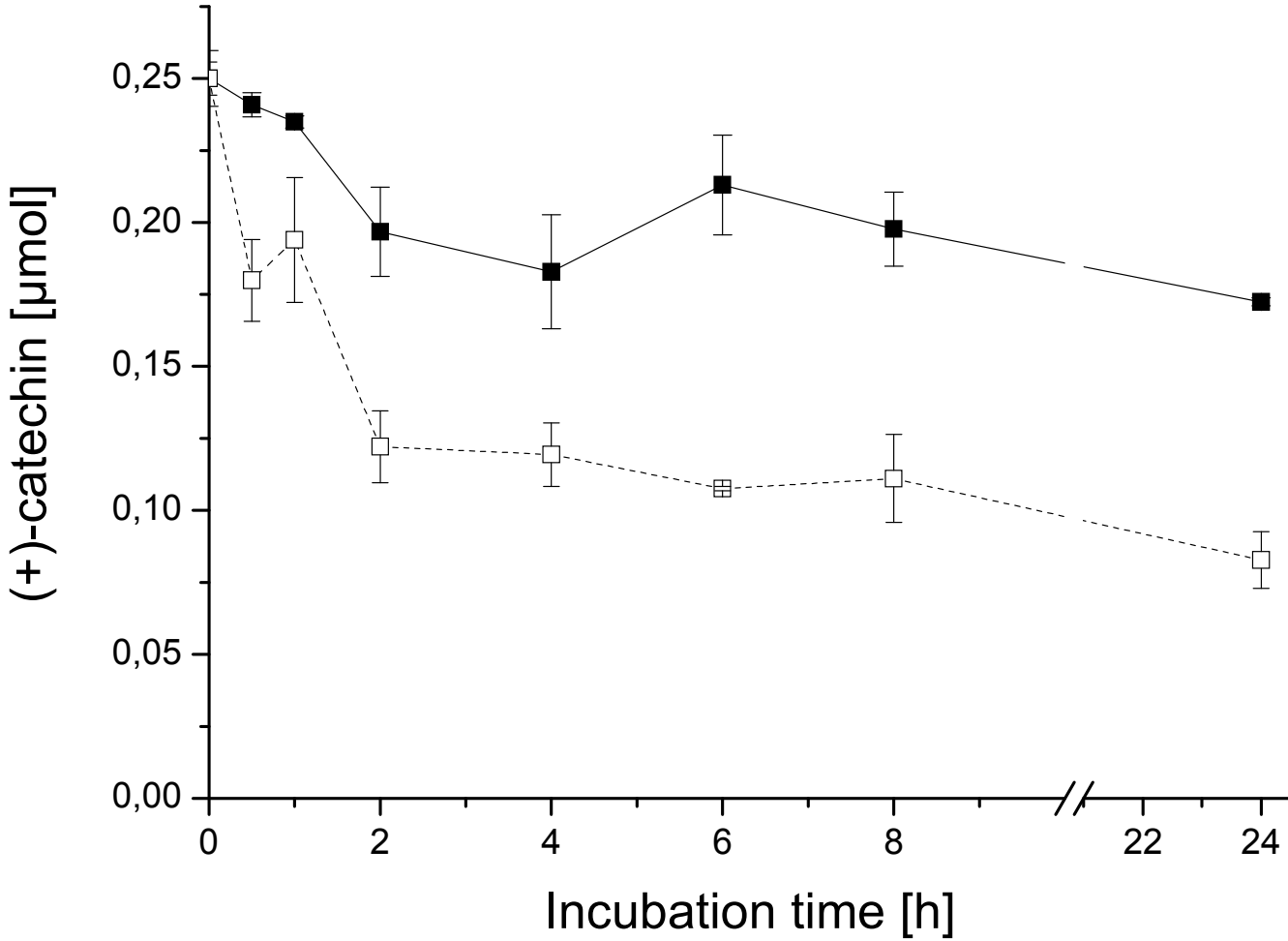
gallic acid (GA)



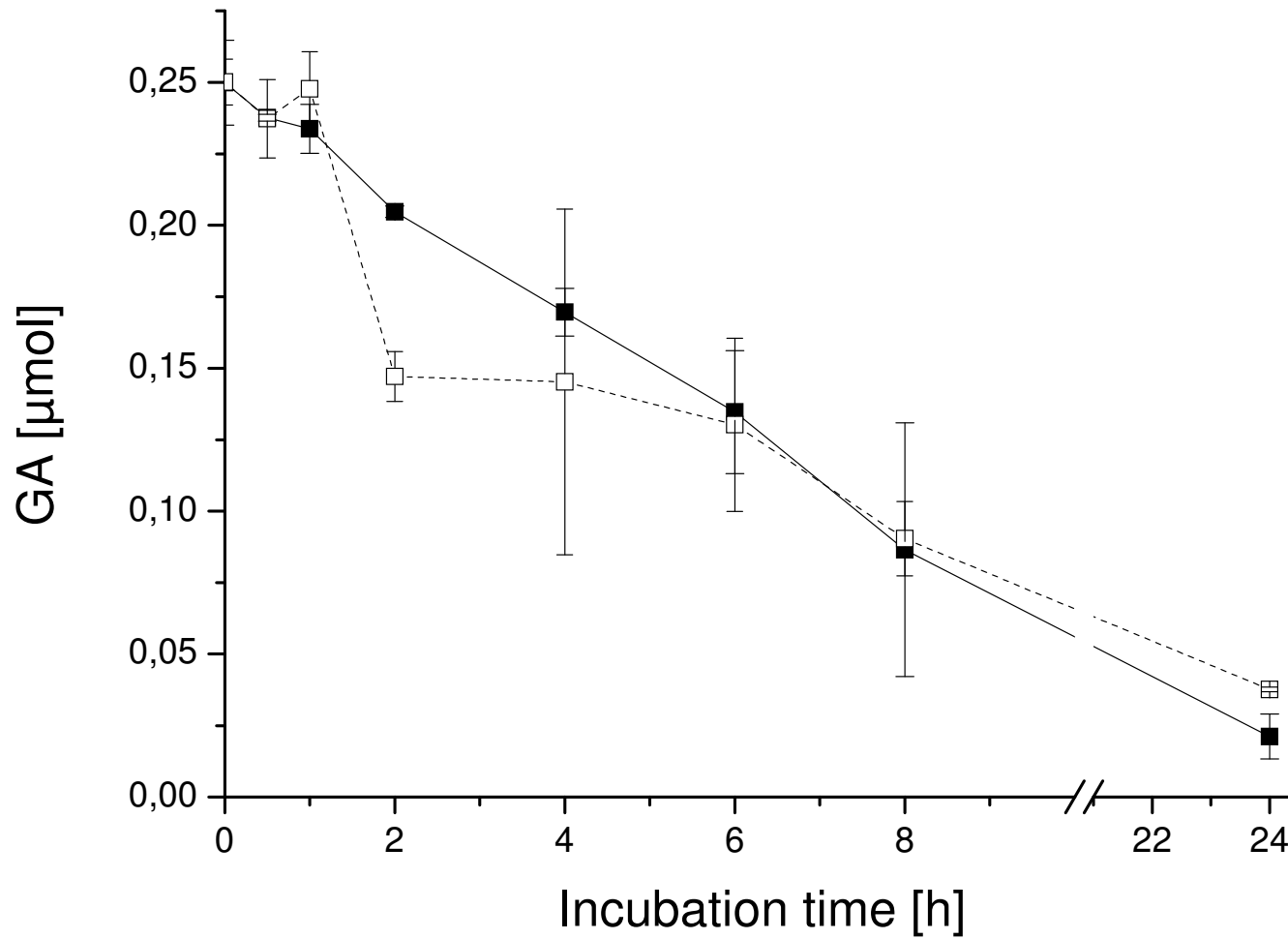


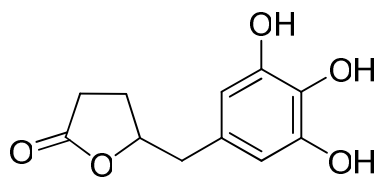
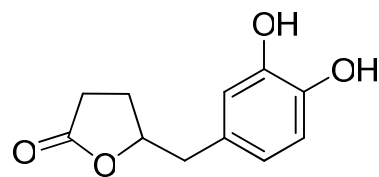
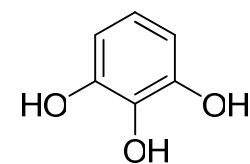






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3',4',5'-trihydroxyphenyl- γ -valerolactone3',4'-dihydroxyphenyl- γ -valerolactone

pyrogallol