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ORIGINAL CONTRIBUTION

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Factors affecting the conversion of apple polyphenols to phenolic acids and fruit matrix to short-chain fatty acids by human faecal microbiota *in vitro*

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Abstract Proanthocyanidins (PAs) in apples are condensed tannins comprised mostly of (−)-epicatechin units with some terminal (+)-catechins. PAs, especially those having a long chain-length, are absorbed in the upper intestine only to a small extent and are passed to the colon. In the colon they are subjected to microbial metabolism by colonic microbiota. In the present article, the ability of human microbiota to ferment apple PAs is studied. Freeze-dried fruit preparations (apple, enzymatically digested apple, isolated cell-walls, isolated PAs or ciders) from two varieties, Marie Ménard and Avrolles, containing PAs of different chain lengths, were compared. Fermentation studies were performed in an *in vitro* colon model using human faecal microbiota as an inoculum. The maximal extent of conversion to known microbial metabolites, was observed at late time point for Marie Ménard cider, having short PAs. In this case, the initial dose also contributed to the extent of conversion. Long-chain PAs were able to inhibit the *in vitro* microbial metabolism of PAs shown as low maxima at early time points. Presence of isolated PAs also suppressed SCFA formation from carbohydrates as compared with that from apple cell wall or faecal suspension without substrates. The low maximal extents at early time points suggest that there is a competition between the inhibitory effect of the PAs on microbial activity, and the ability to convert PAs by the microbiota.

Key words procyanidin – cell-wall – *Malus domestica* Borkh – *in vitro* fermentation – gut microbiota

Introduction

Dietary polyphenols are ubiquitous plant derived secondary metabolites. Proanthocyanidins (PAs) are condensed tannins comprised mainly of (−)-epicatechin units, although some of the terminal units may also be of (+)-catechin [41]. In apples flavan-3-ols (including condensed tannins and monomeric catechins) and hydroxycinnamic acids correspond to the major polyphenol classes in the fruit [18]. The estimated daily intake of flavan-3-ols from human diet varies between 100 and 550 mg [3, 20].

Low amounts of different flavan-3-ol monomers, PA dimers and trimers have been detected in rat urine as sulphate metabolites [44], or in human plasma after consumption of red wine or cocoa as glucuronidated, sulphated and methylated conjugates [21,

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40]. Thus most of the PAs are likely to reach colon and subjected there to microbial conversion. Polyphenols, proanthocyanins among them, can be converted to phenolic acids by colonic microbiota, which increases the occurrence and significance of phenolic acids as one of the major group of circulating metabolites [24]. When polymeric ^{14}C-labeled PAs were incubated with human faecal microbiota *in vitro*, 9–22% of the label was found in the metabolite pool, and ethyl acetate soluble metabolites represented 2.7% of the initial radioactivity [12]. When ^{14}C-labeled PAs were given by gavage to rats, only a small proportion of the label was found in liver (1–1.5%), urine (1–2%) and carbon dioxide (1–2%) [1]. Polymeric PAs were catabolised by human colonic microbiota *in vitro* producing several phenolic acids: 3-hydroxyphenylpropionic acid, 3-phenylpropionic acid, 4-hydroxyphenylpropionic acid and 4-hydroxyphenylacetic acid as major microbial metabolites [12]. Chocolate consumption increased the human urinary excretion of 3-hydroxyphenylpropionic acid, 3,4-dihydroxyphenylacetic acid, 3-hydroxyphenylacetic acid, 3-methoxy-4-hydroxybenzoic acid (vanillic acid) and 3-hydroxybenzoic acid [39]. However, the effect of degree of polymerization on microbial conversion of PAs to phenolic acids has not been studies adequately.

Cell-wall polysaccharides in apples are mainly pectic polysaccharides and cellulose, which enter the colon and are fermented by the microbiota to short-chain fatty acids (SCFA) [8, 31]. There has been studies concerning the fermentability of cell-wall polysaccharides and isolated PAs separately, however, these components are consumed together in the actual fruit and are known to bind spontaneously to each other [25]. This association may cause differences in the microbial metabolism, as described by Aprikian et al. [2], when the preparations are introduced together than if they are administered separately. Furthermore, butyrate, one of the SCFA, has been associated with local beneficial effects on colon health: improved cell proliferation and induction of apoptosis enhancing healthy tissue turnover [23].

Therefore this work aims to compare microbial metabolism of PAs and cell-wall polysaccharides alone and in combination. PA conversion to phenolic acids is also studied from natural apple or cider matrices. The products were selected to elucidate the effect of PA chain length on the fermentation by human gut microbiota.

Materials and Methods

Materials

Apples (*Malus domestica* Borkh) of the Marie Ménard and Avrolles varieties were obtained from the experimental orchard of Institute Francais de Productions Cidricoles (Sées, Orne, France) in 2005. Several apple materials (crude apple powder, enzymatically pre-digested apple powder, purified cell-walls, purified PA extracts and freeze-dried cider) were prepared from fruits of these two distinct apple varieties.

Reagents for analysis of the microbial metabolites of flavanols were as follows: Heptadecanoic acid and succinic acid-2,2,3,3-d_4 used as the internal standard, was purchased from Sigma-Aldrich Inc., (St. Louis, USA) and the following compounds were used as standards: benzoic acid (BA), 3-hydroxybenzoic acid (3-OHBA), 3-(4-hydroxyphenyl)propionic acid (4-OHPPr) and 3-(3,4-dihydroxyphenyl)propionic acid (3,4-diOHPPr) were products from Aldrich, (Steinheim, Germany). 4-Hydroxybenzoic acid (4-OHBA), 2-(3-hydroxyphenyl)acetic acid (3-OHPAc) and 2-(3,4-dihydroxyphenyl)acetic acid (3,4-diOHPAc) were purchased from Sigma (St. Louis, USA). 3-Phenylpropionic acid (3-PPr) and 3,4-dihydroxybenzoic acid (3,4-diOHBA), were from Fluka (Buchs, Switzerland) and 3-(3-hydroxyphenyl)propionic acid (3-OHPPr) was purchased from Alfa Aesar (Karlsruhe, Germany). *N*-Methyl-*N*-trimethylsilyl-trifluoracetamide (MSTFA) from Pierce (Rockford, USA) was used as the silylation reagent.

Methods

Preparation of apple products

The preparation steps of apple samples are described in Fig. 1. Apple powders were prepared from a batch

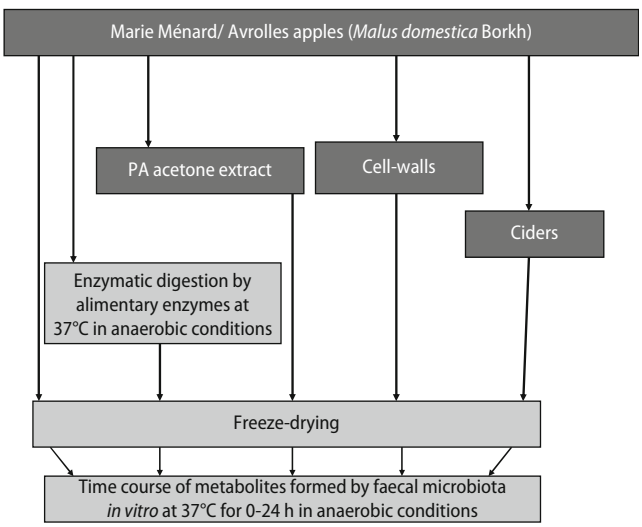

Fig. 1 Schematic diagram of preparation of apple samples prior to *in vitro* fermentation with human faecal microbiota

of 10 fruits as described by Renard et al. [38]. PA acetone extracts were prepared by successive methanol and aqueous acetone extractions from the freeze-dried apple powders (150 g) according to Sanoner et al. [41] and purified as described in Guyot et al. [17]. Cell-walls for the fermentation experiments were isolated from peeled fresh apples of the both varieties by the phenol-buffer method described in Renard [36]. Ciders were prepared from apple juices from 15 kg of Avrolles or Marie Ménard apples by crushing and pressing the fruits. The juices were clarified enzymatically by using polygalacturonase and pectinmethylesterase enzyme mixture (Rapidase C80L; DSM Food, Séclin, France) and microfiltrated (polyvinylidine difluoride membrane, 0.45 mm, Millipore Pellicon, Millipore Corp.) as described by Hubert et al. [22]. The juices were fermented with dried *Saccharomyces uvarum* SRC-73 strain with a dose of $5{\cdot}10^5$ cfu/ml until the density of the juice was below 1,000 g/l. The two cider batches (2 l) were concentrated under vacuum to remove ethanol and freeze-dried.

In vitro enzymatic digestion of apple samples

Enzymatic *in vitro* digestion [4] was performed under anaerobic conditions at 37°C with magnetic stirring (250 rpm) for the freeze-dried apple samples. Mouth (neutral), stomach (acidic) and duodenum (neutral) were mimicked by successive additions of porcine enzymes from Sigma (St. Louis, USA): salivary α-amylase (mouth), pepsin (stomach stage) and pancreatin, bile and mucin (duodenum), respectively. Samples after digestion were washed with distilled water under anaerobic conditions, rapidly frozen using liquid nitrogen and freeze-dried prior to fermentation in the *in vitro* colon model. Apple cell-wall preparations, freeze-dried ciders or PA extracts were not digested enzymatically (Fig. 1).

Analysis of apple samples

Polyphenols in crude apple powders (before and after *in vitro* digestion, BD and AD, respectively) and in the ciders were analyzed after thiolysis by reversed phase HPLC according to Guyot et al. [16]. The purified PA extracts were similarly analyzed according to Guyot et al. [17]. Simple sugars (glucose, fructose and sucrose) were measured colorimetrically using the Boehringer analysis kit (R-Biopharm, St Didier au Mont d'or, France). Alcohol insoluble solids (AIS) from freeze-dried apples and digested apples were prepared according to Renard [36] using 70% ethanol extraction and submitted to pre-hydrolysis in 13 M sulphuric acid (1 h, room temperature) [43]. Ciders and polyphenol extracts were directly submitted to polysaccharide analysis. Myo-inositol was used as internal standard and the individual neutral sugars were analyzed by gas chromatography as alditol acetates [13]. Uronic acids were determined after acid hydrolysis (Seaman procedure) of cell walls by spectrophotometric *m*-hydroxydiphenyl assay described with galacturonic acid as external standard by Blumenkrantz and Asboe-Hansen [9]. Complex carbohydrates were quantified as sugars detected after acid hydrolysis in the AIS, and monomeric and polymeric carbohydrates were expressed as total amounts of monomeric sugar units (μmol/g apple sample).

In vitro fermentation of apple products

Fermentation experiments were performed under strictly anaerobic conditions according to Aura et al. [5, 6] using 10% (w/v) faecal suspension based on a method described by Barry et al. [8]. Faecal suspension was prepared using phosphate buffer (pH 5.5) by pooling the faeces of four (Experiment 1) or five (Experiment 2) healthy donors. Suspension was then diluted to the concentrations above and applied to the samples (100 or 25 mg). Three replicate samples were incubated in a water bath at 37°C for 0, 1, 2, 4, 6, 8 and 24 h and stirred magnetically (250 rpm). Four 2 ml aliquots were drawn from the bottles and microbial metabolites and SCFA were analyzed.

Analysis of the microbial metabolites

Microbial metabolites of phenolic compounds were analyzed using heptadecanoic acid and succinic acid-2,2,3,3-d_4 as internal standards by GC-MS using selective-ion-mode (SIM) after extraction twice with 3 ml ethyl acetate, evaporation of the solvent and subsequent silylation as follows: Dichloromethane (100 μl) and MSTFA (30 μl) was added to the samples, and incubated (5 min, 50°C) [7]. The metabolite formation was calculated as μmol of formed metabolite at each time point per 10 ml. The extent of metabolite formation (%) was calculated from Eq. 1,

$$\text{Extent}_{\text{Substrate}(t)} = \frac{\sum \{\text{Metabolite}_{\text{Substrate}}(t) - \text{Metabolite}_{\text{Feacal control}}(t)\}[\mu\text{mol}]}{\text{Total PA content } [\mu\text{mol}]} \times 100\% \quad (1)$$

in which t = time point; Σ = sum of the following metabolites: 3,4-diOHPPr, 3-OHPPr, 3-PPr, BA, 3,4-diOHPAc, 3-OHPAc, metabolites, which differed from the faecal background in the presence of substrate; PA: Proanthocyanidin. SCFA were analyzed by GC after diethylether extraction according to Aura et al. [6]. Total SCFA formation was a sum of acetic, propionic and butyric acids. The relative proportions of the individual SCFA was calculated in respect to the total SCFA formation at time point 24 h. All results were expressed as averages and standard deviations from three replicate measurements.

Results

Effect of enzymatic digestion on PAs and carbohydrates

The characterisation of the phenolic compounds from Marie Ménard and Avrolles apples powders before (BD) and after (AD) enzymatic digestion, cell-wall polysaccharides, PA extracts and ciders is described in Table 1. The total flavan-3-ol contents and caffeoylquinic acid contents were expressed, because they were known precursors of phenylpropionic acid derivatives. The apple PAs consisted mainly of (−)-epicatechin units regardless of the variety or the product. The gap between total polyphenol content and the sum of flavan-3-ols and caffeoylquinic acid was very small in almost all the apple samples, except for ciders, which also contained *p*-coumaroylquinic acid 1.08 and 2.04 mg/g d.w. in Marie Ménard and Avrolles, respectively. The other significant components were phloridzin (0.60 and 0.98 mg/g d.w.; Table 1) and phloretin xyloglucoside (0.70 and 2.00 mg/g d.w.) in Marie Ménard and Avrolles ciders, respectively. However, their microbial metabolites are not known and thus they were not included in the precursors of phenolic acid metabolites. The cell-wall preparations did not contain precursors of microbial phenolic acid metabolites.

The apple varieties had been chosen to present different average degree of polymerization (*a*DPn). Marie Ménard apple products contained short PA chains (*a*DPn 2.2–9.5), while Avrolles apples contained long polymers (*a*DPn 7.4–71.2). The PA extract showed a higher *a*DPn than the apple material as the monomers

Table 1 Composition of apple powders (BD before and AD after digestion), cell-wall preparations, PA extracts and ciders

	Marie Ménard					Avrolles				
	Apple powder					Apple powder				
	BD	AD	Cell-walls	PA extract	Cider	BD	AD	Cell-walls	PA extract	Cider
Polyphenol content mg/g dry weight apple sample										
Total flavan-3-ols	31	25	ND	777	35	24	56	ND	883	1
Caffeoylquinic acid	6	1	ND	35	31	1	0	ND	6	5
Coumaroylquinic acid	0	0	ND	ND	1	1	0	ND	0	2
Phloridzin	0	0	ND	ND	1	1	0	ND	ND	1
Phloretin xyloglucoside	0	0	ND	ND	1	1	0	ND	ND	2
Total polyphenols	38	26	ND	817	68	28	57	ND	903	11
Characteristics of PAs										
Average degree of polymetization (aDPn)	4.3	8.2	NA	9.5	2.2	35.2	71.2	NA	35	7.4
Catechin units (%)	3	2	NA	2	2	0	0	NA	0	Traces
(−)-Epicatechin units (%)	97	98	NA	99	98	100	100	NA	100	100
Soluble carbohydrates in apple sample										
Fructose (mg/g)	307	29	NA	NA	NA	226	27	NA	NA	NA
Glucose (mg/g)	92	74	NA	NA	NA	94	54	NA	NA	NA
Sucrose (mg/g)	208	58	NA	NA	NA	153	27	NA	NA	NA
Soluble carbohydrates (μmol/g)	3,433	911	NA	NA	NA	2,673	608	NA	NA	NA
Polymeric carbohydrates (mg/g apple sample)	215	545	1,000	1,000	1,000	116	627	1,000	1,000	1,000
Carbohydrate composition (mg/g polymers)										
Rhamnose	9	6	9	1	3	11	6	11	0	3
Fucose	9	8	9	0	0	12	6	12	0	0
Arabinose	130	123	130	3	2	106	99	106	2	0
Xylose	52	67	52	1	13	52	58	52	2	8
Mannose	27	6	27	1	7	26	5	26	0	8
Galactose	46	52	46	2	5	60	70	60	1	4
Glucose	304	286	304	16	159	281	347	281	18	111
Galacturonic acid	194	105	194	1	4	229	128	229	0	4
Polymeric carbohydrates (μmol/g)	1,120	2,425	5,209	172	1,265	611	3,006	5,269	154	919

aDPn average degree of polymerization of total flavan-3-ols, including monomeric flavan-3-ols

and small oligomers had been extracted in the discarded methanol fraction. The lowest *a*DPn were found in the ciders. The total polyphenol content decreased in Marie Ménard apples after enzymatic digestion and separation of solids, whereas it increased in corresponding Avrolles apples after enzymatic digestion and removal of soluble components (Table 1).

Freeze-dried apple samples contained soluble sugars, the amount of which decreased after digestion and washing the residue. The alcohol insoluble fraction (AIS) increased in the enzymatic digestion, which was expected. Cell-walls were purified from polyphenols and soluble carbohydrates, and contained only alcohol insoluble polymeric carbohydrates. Cider samples were applied as such into the colon model, because most of the digestible carbohydrates were already fermented in the cider making process.

Profiles of microbial metabolites

The apple samples (Fig. 1) were fermented with fresh human faecal inoculum from different donors in two experiments, one for each variety using doses indicated in Table 2. The microbial metabolite profiles are shown for Marie Ménard apple samples (Experiment 1) in Fig. 2 and for Avrolles series (Experiment 2) in Fig. 3. Metabolite dynamics can be observed from Marie Ménard experiment (Fig. 2) better than from Avrolles apple experiment (Fig. 3), because Marie Ménard samples showed distinctively higher amounts of microbial metabolites than corresponding Avrolles samples. Primary metabolite (3,4-diOHPPr, Fig. 2a) is formed as a result of ring-fission of catechins and it shows a high profile only in samples containing purified PAs or Marie Ménard cider. Secondary metabolites, 3-OHPPr and 3-PPr, (Figs. 2b and 3-PPr; Fig. 2c) are dehydroxylation products of the first metabolite. Some differences in benzoic acid (BA) and phenylacetic acid metabolites can also be observed between non-digested and digested Marie Ménard apple samples (Fig. 2d–f). In contrast to phenylacetic acids, BA was released or formed from pre-digested Marie Ménard apple more than from non-digested apples. Cider, having the shortest chain length PAs (Table 1), showed highest profiles of phenylpropionic and phenylacetic acid, whereas BA profile from cider was below that of the digested apple. Avrolles cider and samples containing PA extract showed higher phenylpropionic acid profiles than corresponding apple samples (Fig. 3b, c), whereas formation of BA was more pronounced in the samples containing Avrolles PA extract (Fig. 3d). When cell wall polysaccharides and PA extract were fermented together in Avrolles series, the microbial metabolite profiles were in accordance with the purified PA extract (Fig. 3a–f). The postulated pathway of microbial metabolism of apple PA is presented in Fig. 4, including the possible formation of benzoic acid from phenylpropionic acids.

Extent of microbial metabolism of PAs

The doses of samples applied to the *in vitro* colon model, their total amount of microbial metabolite

Table 2 Total amount of precursors of phenolic metabolites and carbohydrates per dose substrate in the *in vitro* colon model and maximal extents and time point of maxima of microbial metabolism

Substrates	Dose (mg)	Phenolic precursors[a] (μmol per dose)	Carbohydrates (μmol per dose)	Maximal extent[b] (% of precursors)	Time point of maxima (h)
Experiment 1					
Marie Ménard					
Apple powder	100	13.1	455	14	8
Digested apple powder	100	9.0	334	10	6
Cell-walls	100	0.0	521	ND	ND
PA extract	25	72.5	4	3	2
Cider	100	21.1	127	44	24
Experiment 2					
Avrolles					
Apple powder	100	9.0	328	5	8
Digested apple powder	100	19.9	361	2	0
Cell-walls	100	0.0	527	ND	ND
PA extract	25	79.3	4	2	4
PA extract + cell-walls	25 + 100	79.3	531	2	8
Cider	100	1.7	92	62	4

[a]Sum of total flavan-3-ols (MW 290 g/mol) and chlorogenic acid (MW 354 g/mol), the known precursors of phenylpropionic acid derivatives and benzoic acid
[b]Calculations according to Eq. 1. Sum of 3-(3,4-dihydroxyphenyl)propionic acid, 3-(3-hydroxyphenyl)propionic acid, 3-phenylpropionic acid, benzoic acid, 2-(3,4-dihydroxyphenyl)acetic acid and 2-(3-hydroxyphenyl)acetic acid. Experiments were performed using a pool of faeces from 4 or 5 donors in experiment 1 and 2, respectively

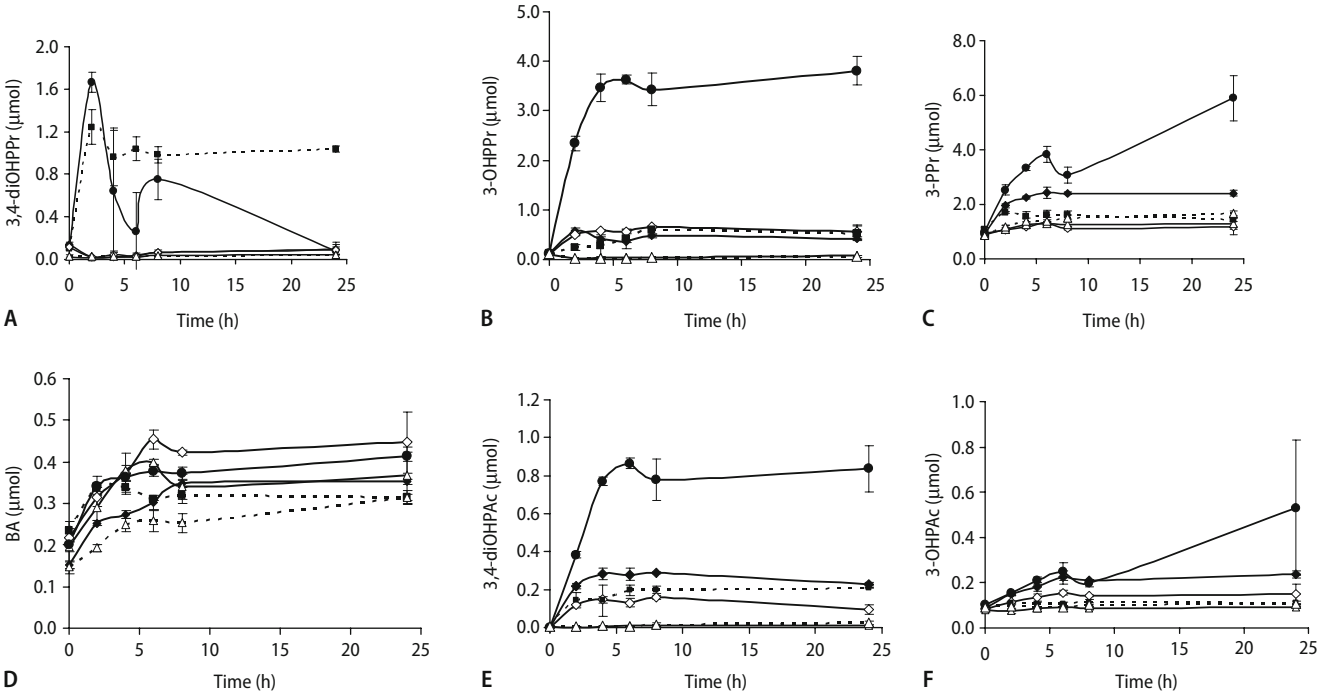

Fig. 2 Microbial metabolite profiles of precursors (total flavan-3-ols and chlorogenic acid) from Marie Menard apple samples. **a** 3-(3,4-Dihydroxyphenyl)propionic acid (3,4-diOHPPr); **b** 3-(3-Hydroxyphenyl)propionic acid (3-OHPPr); **c** 3-Phenylpropionic acid (3-PPr); **d** Benzoic acid (BA); **e** 2-(3,4-Dihydroxyphenyl)acetic acid (3,4-diOHPAc) and **f** 2-(3-Hydroxyphenyl)acetic acid (3-OHPAc). Symbols: Apple (*dashed with filled diamond*); Digested apple (*dashed with open diamond*); Cell-walls (*dashed with open triangle*); PA (*dotted with filled square*); Cider (*dashed with filled circle*); No substrate (*dotted with open triangle*)

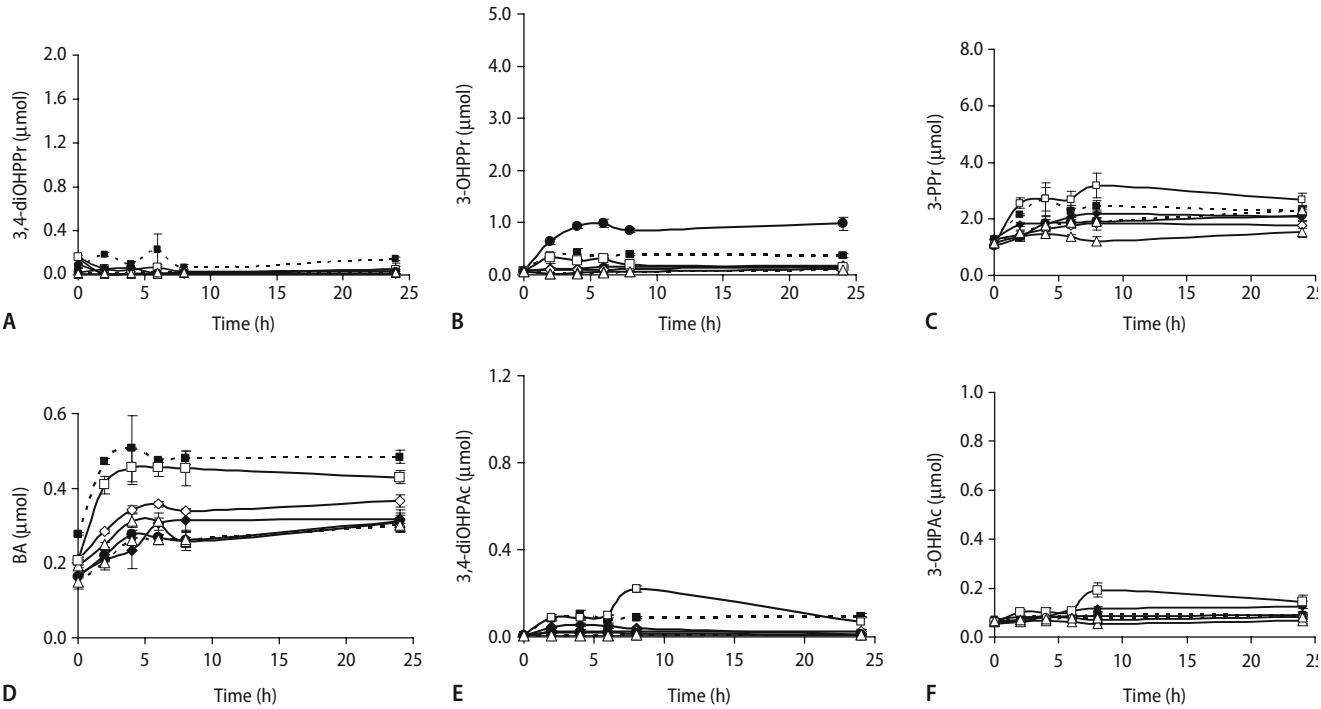

Fig. 3 Microbial metabolite profiles of precursors (total flavan-3-ols and chlorogenic acid) from Avrolles apple samples. **a** 3-(3,4-Dihydroxyphenyl)propionic acid (3,4-diOHPPr); **b** 3-(3-Hydroxyphenyl)propionic acid (3-OHPPr); **c** 3-Phenylpropionic acid (3-PPr); **d** Benzoic acid (BA); **e** 2-(3,4-Dihydroxyphenyl)acetic acid (3,4-diOHPAc) and **f** 2-(3-Hydroxyphenyl)acetic acid (3-OHPAc). Symbols: Apple (*dashed with filled diamond*); Digested apple (*dashed with diamond*); Cell-walls (*dashed with triangle*); PA (*dotted with filled square*); PA and cell walls (*dashed with open square*); Cider (*dashed with filled circle*); No substrate (*dotted with open triangle*)

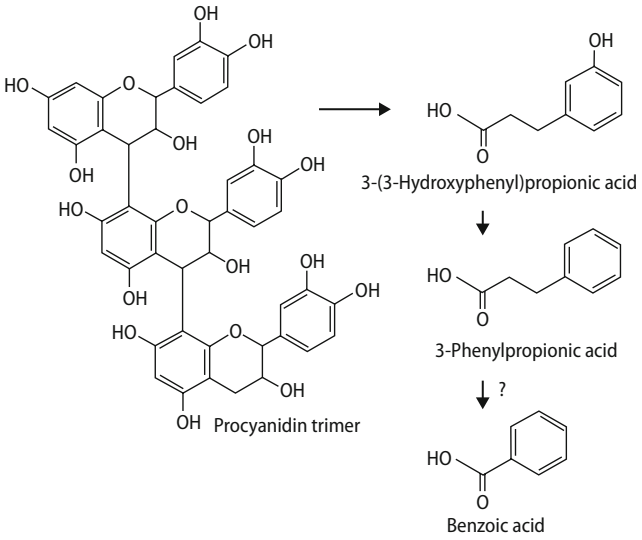

Fig. 4 Postulated microbial metabolites from apple PAs

precursors (a sum of total amount of flavan-3-ols and caffeoylquinic acid in μmol per dose) and the total amount of sugar units (μmol per dose) are described in Table 2. Extents of conversion were calculated taking into account the six microbial metabolites, which showed distinctive difference from faecal background in the presence of substrates (3,4-diOHPPr, 3-OHPPr, 3-PPr, BA, 3,4-diOHPAc, 3-OHPAc), and the total flavan-3-ol and caffeoylquinic acid contents of the samples subjected to colonic microbiota *in vitro*. Extents were calculated for all the time points as described in the Eq. 1 (results not shown) and maximal extents and their time points are described in Table 2. Metabolite profiles showed the highest concentration of most of the metabolites for ciders, resulting in high maximal extent of conversion 44 and 62%, for Marie Ménard and Avrolles, respectively (Table 2). Avrolles cider sample showed a higher extent of conversion than Marie Ménard cider, but also lower amounts of precursors.

Maximal extent of conversion was reached much later (at 6–8 hours) with Marie Ménard apple samples than with corresponding PA extract (2 h) (Table 2). The PA extracts were metabolised to the lowest extent and their conversion to phenolic acids was stopped early (2 and 4 hours, for Marie Ménard and Avrolles, respectively). However, when Avrolles PA extract was fermented in the presence of cell-wall preparation, the conversion time was doubled to 8 h compared with that of PA extract alone without large extension of conversion efficiency (Table 2). Both cell-wall preparations were devoid of phenolics and no phenolic acid metabolites were formed, when they were fermented alone by faecal microbiota.

SCFA formation

Avrolles apple samples showed larger differences in the SCFA formation than corresponding Marie Ménard samples, but the trends were similar (Fig. 5a, c): Non-digested apple had higher fermentation rate than the digested sample. Cell-wall preparations showed the same rate and extent of SCFA formation as digested apple samples. Furthermore, Avrolles cider also differed more in SCFA formation than Marie Ménard cider as compared with the corresponding SCFA formation profiles of the fruit matrices. Presence of PA extract was also able to suppress SCFA formation as compared with faecal control or with cell wall preparation alone (Fig. 5a, c).

Relative proportions of SCFA showed highest relative proportion of acetate, intermediary for butyrate and lowest for propionate in all apple samples regardless of the variety. Non-digested Marie Ménard apples and cider showed the highest relative proportions of butyrate as compared with other apple samples. However, the same result was not that evident for Avrolles cider due to high standard deviations between replicates (Fig. 5b, d).

Discussion

Effect of enzymatic digestion on composition of apple samples

The effect of the enzymatic digestion and removal of soluble components on total flavan-3-ol content was dependent on the chain length of PAs. When the chain length was short as in Marie Ménard apples, the total flavan-3-ol content decreased and the average degree of polymerization (*a*DPn) increased after rinsing, indicating loss of short-chain flavan-3-ols. As *a*DPn of Avrolles apple flavan-3-ols was already high, after isolation of non-digestible solids the PAs were even longer. Removal of soluble components increased the relative content of long-chain PAs. The PA chain length in ciders was shorter than in apples as a contribution of the affinity of long-chain PAs for apple cell-walls [25]. The same phenomenon affects extraction to water during the rinsing procedure after the enzymatic digestion. The gap between the amounts of total polyphenols and those of total flavan-3-ols and caffeoylquinic acid decreased in the digestion, reflecting removal of soluble monomeric phenolic compounds. Cell-wall preparations, PA extracts or ciders were not enzymatically digested, because removal of soluble components after the digestion procedure would not have been possible without losses of components under investigation.

Apple cell-wall AIS fractions had roughly the same carbohydrate composition before and after enzymatic

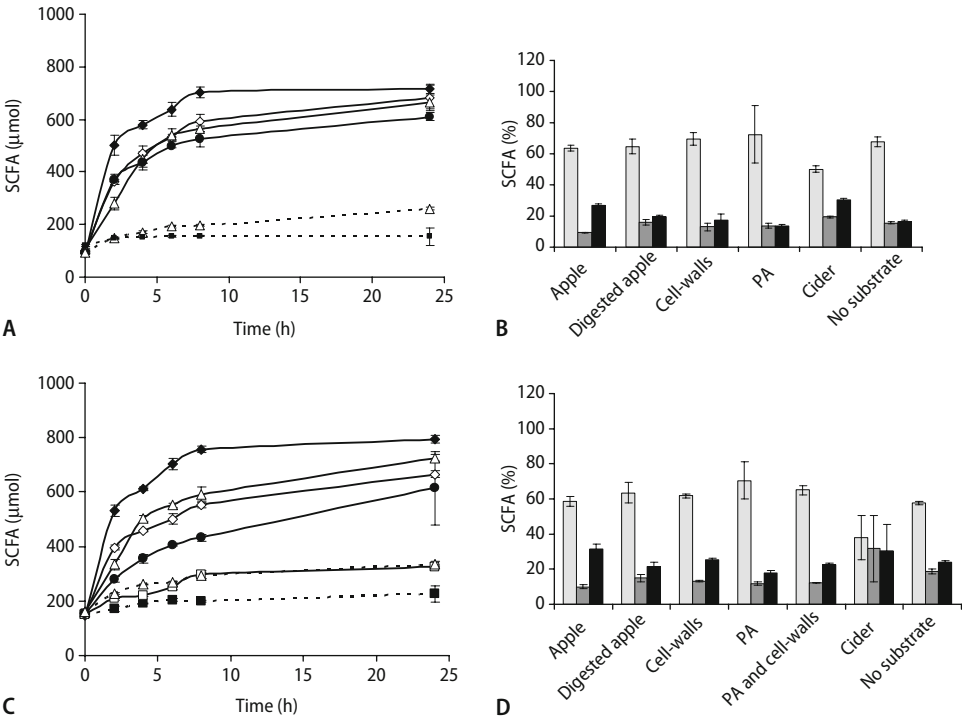

Fig. 5 (**a**) Total short-chain fatty acid (SCFA) formation (µmol) and (**b**) relative proportions of SCFA (%) in samples containing Marie Ménard apple products; (**c**) Total SCFA formation (µmol) and (**d**) relative proportions of SCFA (%) in samples containing Avrolles apple products.Total SCFA contents are the sums of acetic (*less densed dots*), propionic (*medium densed dots*) and butyric (*dots with black background*) acid contents in the samples and the relative proportions of individual SCFA were calculated as % of total SCFA. Symbols: Apple (*dashed with filled diamond*); Digested apple (*dashed with open diamond*); Cell-walls (*dashed with open triangle*); PA (*dotted with filled square*); PA and cell walls (*dashed with open square*); Cider (*dashed with filled circle*); No substrate (*dotted with open triangle*)

digestion. The polysaccharides present in apple cell-walls are pectins (galacturonic acid, arabinose, galactose, rhamnose), cellulose (glucose) and xylogalacturonans (xylose, non- cellulosic glucose, galactose, fucose) [36]. In this study, some of the pectic polysaccharides were lost during digestion, shown as decrease in galacturonic acid. The loss was evident in both Avrolles and Marie Ménard apples.

Relevance of dose in the *in vitro* fermentation

Daily intake of PAs varies according to the proposed source: Auger et al. [3] reported that with moderate consumption of red wine (180 ml/day), containing 100.4 mg (0.35 mmol) flavanol units. Hammerstone et al. [20] estimated that the daily intake per serving of food could vary from 147.1 to 164.7 mg (0.51–0.57 mmol flavanol units). A moderate red wine consumer, who eats three servings of food daily, could have approximately 100–550 mg of PAs (0.3–1.9 mmol flavanol units). According to Guyton and Hall [19] 1,500 ml of chyme is emptied daily to caecum from ileum. Thus the average calculated concentration of PAs entering the caecum could vary between 0.2 and 1.3 µmol/ml. In the present study the dose of phenolic acid precursors varied from 1.7 to 79.3 µmol per 10 ml of faecal suspension corresponding 0.17–7.9 µmol/ml in the *in vitro* colon model. Thus the doses of PAs from fruit samples in the experiments were of the same magnitude as the possible physiological concentrations could be as calculated from the possible daily intake data.

Identification of the microbial metabolites

The anticipated microbial metabolites of apple samples were phenylpropionic acid derivatives (3,4-di-OHPPr, 3-OHPPr and 3-PPr), benzoic acid (BA) from flavan-3-ols (Fig. 4) and hydroxycinnamic acids and acetic acid derivatives (3,4-diOHPAc and 3-OHPAc) from flavonols [5]. Deprez et al. [12] showed that the main microbial metabolites of synthesized labeled PAs were 3-OHPPr, 4-OHPPr, 4-OHPAc and 3-PPr. In our study 4-OHPPr concentrations in apple samples did not differ from those in the faecal background and 4-OHPAc was not among the standards. Caffeoylquinic acid can be converted to 3-OHPPr, which can be metabolized to benzoic acid via β-oxidation [15]. Thus caffeoylquinic acid metabolism by gut microbiota can contribute to the concentrations of these microbial metabolites. These results are in accordance with the data concerning urinary metabolites *in vivo* after intake of cocoa PAs [39], but naturally hepatic metabolites such as hippuric acid (glycinated BA) or methoxylated phenolic acids, are not present in *in vitro* faecal incubations. The microbial metabolites of the minor phenolic compounds in cider, phloretin and phloridzin (phloretin-2-O-glucoside) are not to our knowledge known. However, phloretin is formed from two aromatic hydroxylated rings coupled with

$CO–CH_2–CH_2$-bridge [26] and it could be speculated that phloretin could contribute to the formation of hydroxyphenylacetic acid and benzoic acid, if only oxygen ether bond of the bridge is broken similarly as ester bonds are by colonic esterases [34].

It is possible that the low extent of ring-fission metabolites observed in Avrolles series was due to metabolites not detected in the targeted analysis performed by GC-MS. A later study has shown that (+)-catechin and (−)-epicatechin can also be converted mainly to 5-(3,4-dihydroxyphenyl)- and 5-(3-hydroxyphenyl)valeric acid metabolites instead of corresponding hydroxyphenylpropionic acid derivatives, depending on the human microbiota used as an inoculum [7]. Furthermore, (−)-epicatechin and its galloylated derivatives can be converted to hydroxylated valerolactone derivatives [30]. However, valeric acids or valerolactones are not commercially available and thus were missed by the method used in the present study.

Effect of chain length of PAs

Short PAs in cider exhibited a strong metabolite formation during the entire fermentation experiment and higher concentration of PAs could be tolerated without inhibition of metabolite formation. The amount of precursors in Marie Ménard cider was 12 times higher than that in Avrolles cider. The Avrolles cider sample showed a higher apparent extent of conversion than the Marie Ménard cider, but since it contained lower amount of precursors, the extent was more dependent on the low precursor content than high amount of detected metabolites. PAs in ciders were shorter in chain length due to selective extraction of lower molecular weight PAs [11] or due to poorer extractability of long-chain precursors to the cider [25].

The chain length of PAs has a more crucial role in their microbial metabolism than the dose. The calculated extents confirmed the result: the highest maximal extents were achieved, when the conversion to phenolic acids was allowed to last longer, whereas low maximal extents were reached at early time points (2–4 h). Long chain length of PAs in Avrolles samples was shown as low amounts of metabolites. It is worth of note that different donors of faecal samples posses a different intestinal microbiota, which causes high individual variation in concentrations of metabolites [10] and even change in the site of ring-fission [7]. Thus comparisons between different fermentation experiments using different inocula can be made only with great caution.

In the present study the extents of PA conversion were all in all very low for both the PA extracts. This result is in agreement with the previous work with radiolabelled PAs performed by Deprez et al. [12], in which PAs were converted only to a small extent (2.7% of original dose). Furthermore, Gonthier et al. [14] showed in their rat study that the degree of polymerization strongly decreased absorption and both the number of metabolites and extent of microbial metabolism. Scalbert [42] has reported antimicrobial effects and inhibitory action of long-chain PAs on cell-wall degrading enzyme activities. Furthermore, tannins exhibit binding to proteins causing haze in beverages [45] and inhibition of digestive enzymes [29]. Even hydrolysable tannins (ellagitannins) have an ability to inhibit specific bacteria [35] and proanthocyanidins exhibited antimicrobial properties against ruminal bacteria [46].

SCFA

SCFA formation from apple samples and ciders showed similar profiles and extents regardless of the variety. The differences between Marie Ménard apple products were smaller in SCFA production than those between the Avrolles apple products. SCFA profiles of non-digested apples were slightly above those of the digested ones reflecting removal of starch and soluble sugars after enzymatic digestion and rinsing procedures. Solid apple samples containing more polymeric carbohydrates showed expectedly higher amounts of SCFA than ciders. Nevertheless, SCFA formation from ciders show that also beverages have a carbohydrate matrix despite their soluble nature and the fermentation by yeast. SCFA formation from ciders originated most likely from yeast glucans designated as high glucose content.

SCFA formation by colonic microbiota *in vitro* has been shown extensively for various polysaccharides including pectins [8, 31]. The presence of long-chain PAs were consistently able to inhibit the SCFA formation from cell-walls and from carbohydrates originated from the faecal inocula as shown by results from the Avrolles series, a phenomenon which was not apparent, when the PAs were enclosed in the natural apple matrix (Fig. 5a, c). This inhibition is most likely due to inhibition of cell-wall degrading enzymes of the microbiota [42]. The enzymes hydrolyse the polysaccharides to monosaccharides, which is a step required prior to formation of SCFA by the microbiota. The inhibition of cell-wall polysaccharide degradation by microbial enzymes in the presence of PAs is in accordance with the inhibition observed for pectolytic enzymes, which was anticipated to occur either by steric effects or through direct enzyme inhibition [37].

Butyric acid production was not high in digested apple or apple cell-walls, which is in agreement with

literature. Pectic polysaccharides have not shown significantly higher relative proportions of butyrate as compared with other polysaccharide preparations [8, 31]. Dextrans (α-glucan) have been shown to increase the relative proportion of butyrate in *in vitro* faecal fermentation model [32]. Thus higher relative proportion of butyrate may reflect the higher content of glucans in non-digested apples (Table 1) or yeast glucans in ciders [28].

Potential effects of non-fermented PAs

Since the degradation of PAs appears to occur at low extent, it is likely that luminal tannins may cause enzyme inhibition, as described by McDougall and Stewart [29]. Condensed tannin and pectin exhibited an effect on lipid metabolism in the rat, by lowering plasma cholesterol level [27]. PA dimers and oligomers exhibit protective role in obesity and insulin resistance, focusing their role on the adipocytes, where PAs modify lipid synthesis, lipid degradation, glucose uptake, and adipose differentiation [33]. Tomaru and co-workers [47] showed that dietary supplementation of cacao liquor PAs prevented elevation of blood glucose levels in diabetic obese mice dose-dependently, suggesting benefits in preventing the onset of type 2 diabetes mellitus. Because PAs may change the behavior of colonic fermentation, the impact of DF on fermentation or colonic transit may be altered. Further studies are needed to determine the biological significance of PAs and their microbial metabolites.

Conclusions

Chain length is an important factor affecting the microbial conversion of PAs to phenolic acids. Isolated, long-chain PAs were able to suppress both phenolic acid and SCFA formation *in vitro* by faecal microbiota, suggesting inhibitory effects on luminal enzymes.

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