Towards Microbial Fermentation Metabolites as Biomarkers for Health Benefits of Prebiotics

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for working group on Microbial Metabolism and Fermentation

SUPPORTED BY THE ILSI EUROPE PREBIOTICS TASK FORCE
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Manipulation of the microbiota with dietary interventions is a promising target for improvement of host health

Metabolites produced by the gut bacteria contribute to the metabolic phenotype of the host

Analyzing the activity of the microbiota (at the metabolite level) rather than its composition to assess the impact of dietary interventions
Objectives

A literature search was performed to address:

• Available evidence for the beneficial or harmful effects of known microbial metabolites including short chain fatty acids and protein fermentation products.

• The potential for functional analysis of faecal water

• The applicability of metabolome signatures and systems biology
Methods

Summarising available evidence
a. List of relevant metabolites

b. Highlighted metabolites
   • Harmful/beneficial effects
   • Normal ranges
   • Ranges in pathological conditions

c. More holistic approach
   • Functional analysis of faecal water
   • Metabolomics
   • Metagenome analysis
Colonic microbial metabolites

<table>
<thead>
<tr>
<th>Carbohydrate (Dietary fibre)</th>
<th>Protein</th>
<th>Plant Polyphenolics</th>
<th>Fat and related bile acids</th>
</tr>
</thead>
<tbody>
<tr>
<td>• Short chain fatty acids</td>
<td>• Ammonia</td>
<td>• Large range of phenolic compounds and acids including:</td>
<td>• Hydroxy fatty acids</td>
</tr>
<tr>
<td>o Acetate</td>
<td>• Hydrogen Sulphide</td>
<td>• Simple phenols</td>
<td>• Secondary bile acids</td>
</tr>
<tr>
<td>o Propionate</td>
<td>• Phenols</td>
<td>• Glycinated benzoic acids</td>
<td>• Long chain aldehydes</td>
</tr>
<tr>
<td>o Butyrate</td>
<td>• p-cresol</td>
<td>• Derivatives of benzoic acid</td>
<td></td>
</tr>
<tr>
<td>• Lactate</td>
<td>• Indoles</td>
<td>Derivatives of</td>
<td></td>
</tr>
<tr>
<td>• Succinate</td>
<td></td>
<td>o Phenyl acetic acid</td>
<td></td>
</tr>
<tr>
<td>• Alcohols</td>
<td></td>
<td>o Phenylpropionic acid</td>
<td></td>
</tr>
<tr>
<td>• Gasses:</td>
<td></td>
<td>o Mandelic acids</td>
<td></td>
</tr>
<tr>
<td>o Hydrogen</td>
<td></td>
<td>o Cinnamic acids</td>
<td></td>
</tr>
<tr>
<td>o Methane</td>
<td></td>
<td>o Equols</td>
<td></td>
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<tr>
<td>o Carbondioxide</td>
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</tbody>
</table>
Metabolism of SCFA

- Absorption
  - SCFA in colon
  - 90-95%

- Splanchnic extraction
  - Colonocytes
  - Liver
  - acetate: 40-75%
  - propionate: 75-90%
  - butyrate: 90-98%

- Extravascular organs

- Systemic circulation
  - Oxidation to CO₂

- Feces
  - 5-10%

- Urine

- Inaccessibility of appropriate body compartment
  - Leaves faeces, plasma, urine
  - Not representative

- Where / when to measure
- Large normal variations – diet and other factors
Fecal levels of SCFA

- Acetate: 36-60 µmol/g
- Propionate: 11-16 µmol/g
- Butyrate: 8-15 µmol/g
- Total SCFA: 60-90 µmol/g
SCFA levels depend on age

Tjellstrom et al. Microb Ecol Health Dis 2013, 24, 20905
Wang et al. Dig Dis Sci 2012, 57, 2096-2102
De Filippo et al. Proc Natl Acad Sci U S A 2010, 107, 14691-14696
Holscher et al. J Parenter Enteral Nutr 2012, 36, 95s-105s
Norin et al. Microb Ecol Health Dis 2004, 16, 8-12
Samuelsson et al. Diabet Med 2004, 21, 64-67
SCFA in obesity

Microbiota and SCFA in Lean and Overweight Healthy Subjects

Andreas Schwiertz¹, David Taras², Klaus Schäfer³, Silvia Beijer³, Nicolaas A. Bos³, Christiane Donus⁴ and Philip D. Hardt⁴

Increased faecal SCFA excretion could be due to decreased MCT active uptake in high fat/low CHO diets

Bile salt CDCA and *E. coli* EPEC inhibit butyrate uptake

MCT transporters expression and apical location is promoted by luminal SCFA

Borthakur et al. Am J Physiol Gastrointest Liver Physiol 2012, 303, G1126-G1133
In vitro SCFA production from obese and lean microbiota
SCFA levels in disease

• IBD: lower levels of faecal SCFA
• celiac disease: increased levels of total SCFA and acetate
• allergy: lower faecal levels of propionate and butyrate

⇒ causative to disease?
⇒ markers of disease?
SCFA may beneficially impact on mammalian processes

- Apoptosis
- Cellular proliferation in non-cancer cells
- Inflammation
- Recruitment of immune cells to intestine
- Neutrophil activity/oxidative burst
- Adipose tissue – adipokine production, fat storage
- Tight junction control
- Expression of incretins/gut peptides, and regulation of food intake
- Intestinal motility
- Cholesterol production/lipogenesis
- Glucogenesis
- Cellular energy metabolism
- Thermogenesis
- Inhibits tumorigenesis
- DNA miss-match repair

Note: most mechanistic data from *in vitro* or animal studies – rodents, pigs, chickens
Minor organic acids

• Lactate, succinate
  • do not accumulate much in faeces in health
  • cross-feeding between bacteria leads to formation of main SCFA
  • Lactate:
    • considered as a marker of dysbiosis
    • cosubstrate for sulphate reducing bacteria ⇒ promote sulphide generation
  • Succinate:
    • may act as a signal of inflammation
    • Increased levels have been linked to inflammatory bowel diseases (IBD)
# Metabolites of protein fermentation

<table>
<thead>
<tr>
<th>Metabolite</th>
<th>Origin</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ammonia</td>
<td>• Bacterial degradation of amino acids</td>
</tr>
<tr>
<td></td>
<td>• Hydrolysis of urea</td>
</tr>
<tr>
<td>Phenols</td>
<td>• Major metabolites of bacterial fermentation of aromatic amino acids</td>
</tr>
<tr>
<td></td>
<td>• Rapidly absorbed by colonic mucosa and excreted in urine</td>
</tr>
<tr>
<td></td>
<td>• Do not accumulate in healthy subjects</td>
</tr>
<tr>
<td>p-cresol</td>
<td>&lt; tyrosine</td>
</tr>
<tr>
<td>phenol</td>
<td>&lt; phenylalanine</td>
</tr>
<tr>
<td>indole</td>
<td>&lt; tryptophan</td>
</tr>
</tbody>
</table>
Urinary and faecal levels of p-cresol

- Higher urine levels in obese than in normal weight subjects
- Urine p-cresol levels increase in very old subjects

![Graph showing urinary and faecal levels of p-cresol](image)

160-530 µmol/d

0.14-0.60 µmol/g

References:
- Birkett *et al.* Am J Clin Nutr 1996, 63, 766-772
- Damen *et al.* J Nutr 2012, 142, 470-477
- Ling *et al.* J Nutr 1992, 122, 924-930
- Renwick *et al.* Hum Toxicol 1988, 7, 267-272
- De Preter *et al.* Br J Nutr 2004, 92, 439-446
- De Preter *et al.* J Am Coll Nutr 2007, 25, 541-549
- De Preter *et al.* Am J Physiol Gastrointest Liver Physiol 2007, 292, G358-G368
- Cloetens *et al.* Br J Nutr 2010, 103, 703-713
- Windey *et al.* PlosOne 2012, 7, Article Number: e52387
- Gostner *et al.* Br J Nutr 2006, 95, 40
- Adams *et al.* Lancet 1985, 2, 1313-1313
- Heavey *et al.* Br J Nutr 2003, 89, 509-515
Urinary and faecal levels of phenol

Damen et al. J Nutr 2012, 142, 470-477
Ling et al. J Nutr 1992, 122, 924-930
Renwick et al. Hum Toxicol 1988, 7, 267-272
Cloetens et al. Br J Nutr 2010, 103, 703-713
Gostner et al. Br J Nutr 2006, 95, 40
Adams et al. Lancet 1985, 2, 1313-1313
Effects of phenolic compounds

• Effects on epithelial cells mainly determined in *in vitro* incubation tests
  • Decreased viability
  • Reduced epithelial barrier function

• No systemic toxicity in healthy subjects

• Accumulates in serum in chronic kidney disease ⇒ uremic toxin
  ⇒ contributes to endothelial disfunction

• Very limited data on toxicity of indol

Pedersen *et al.* Scand J Gastroenterol 2002, 37, 74-79
Mccall *et al.* Toxicol Appl Pharmacol 2009, 241, 61-70
Hughes *et al.* Nutr Cancer-An Int J 2008, 60, 259-266
Meijers *et al.* Am J Kidney Dis 2009, 54, 891-901
Fecal levels of ammonia

- Comparable levels in overweight and normal weight subjects
- Higher levels in children with autism spectrum disorders (42 µmol/g)

Slavin et al. Food & Function 2010, 2, 72-77
Shinohara et al. Anaerobe 2010, 16, 510-515
Effects of ammonia

• effects on epithelial cells:
  • alters nucleic acid synthesis
  • changes the morphology and intermediary metabolism of intestinal cells
  • reduces the lifespan of cells

• No adverse effects upon oral administration

Blachier et al. Amino Acids 2007, 33, 547-562
### List of microbial catabolites of common plant polyphenols and their putative health effects.

<table>
<thead>
<tr>
<th>Plant polyphenol</th>
<th>Microbial Catabolite</th>
<th>Possible health effects</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>(-)-epicatechin</td>
<td>4-hydroxyphenylacetic acid</td>
<td>Antibacterial activity against Gram-negative enterobacteria via outer membrane destabilization</td>
<td>Alakomi 2007, Ko 2009, Roowi 2010</td>
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<tr>
<td></td>
<td>3,5-dihydroxyphenylpropionic acid</td>
<td>Antimicrobial activity against Gram-negative enterobacteria via outer membrane destabilization</td>
<td></td>
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<tr>
<td>(-)-epigallocatechin</td>
<td>4-hydroxyphenylacetic acid</td>
<td>Antimicrobial and/or antiestrogenic activity in vitro</td>
<td>Roowi 2010</td>
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<td></td>
<td>3,5-dihydroxyphenyl-propionic acid</td>
<td>Antimicrobial and/or antiestrogenic activity in vitro</td>
<td></td>
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<td></td>
<td>Pyrogallol</td>
<td>Antimicrobial activity against Gram-negative enterobacteria via outer membrane destabilization</td>
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<td>4-hydroxyphenylacetic acid</td>
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<td></td>
</tr>
<tr>
<td>Daidzein</td>
<td>Equol</td>
<td>Phyto-oestrogen important for heart and bone health, and possible colon cancer protectants</td>
<td>Jackman 2007, Ishimi 2009, Davis 2009, Salma 2009</td>
</tr>
<tr>
<td>Quercetin</td>
<td>O-demethylangolensin</td>
<td>Estrogenic and/or antiestrogenic activity</td>
<td>Larrosa 2006, Salma 2009</td>
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<td></td>
<td>2,3,4-dihydroxyphenylacetic acid</td>
<td>Estrogenic and/or antiestrogenic activity</td>
<td></td>
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<td></td>
<td>3,4-dihydroxyphenylpropionic acid</td>
<td>Estrogenic and/or antiestrogenic activity</td>
<td></td>
</tr>
<tr>
<td></td>
<td>3-(3',4'-dihydroxyphenyl)propionic acid</td>
<td>Estrogenic and/or antiestrogenic activity</td>
<td></td>
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<tr>
<td>Kaempferol</td>
<td>Phloroglucinol</td>
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<tr>
<td>Naringenin</td>
<td>3-(4-hydroxyphenyl)propionic acid</td>
<td>Antimicrobial activity against Gram-negative enterobacteria via outer membrane destabilization</td>
<td>Salma 2009</td>
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<tr>
<td></td>
<td>Phloroglucinol</td>
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<tr>
<td>Isoxanthohumol</td>
<td>B-prenylnaringenin</td>
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<tr>
<td>Catechin and epicatechin</td>
<td>3-(3',4'-dihydroxyphenyl)propionic acid</td>
<td>Antimicrobial activity against Gram-negative enterobacteria via outer membrane destabilization</td>
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<td></td>
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<tr>
<td>Ellagitannins/ellagic acid</td>
<td>Urolithin A</td>
<td>Estrogenic and/or antiestrogenic activity, antimalarials</td>
<td>Del Rio 2010, Larrosa 2006, DeFaggi 2010</td>
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<td></td>
<td>Urolithin B</td>
<td>Estrogenic and/or antiestrogenic activity, antimalarials</td>
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<td></td>
<td>Urolithin C</td>
<td>Estrogenic and/or antiestrogenic activity</td>
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<td></td>
<td>Urolithin D</td>
<td>Estrogenic and/or antiestrogenic activity</td>
<td></td>
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<tr>
<td></td>
<td>3,4-dihydroxybenzoic acid</td>
<td>Rutin and catabolites inhibit AGE formation in vitro; Antimicrobial activity against Gram-negative enterobacteria via outer membrane destabilization</td>
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<tr>
<td>Lignans</td>
<td>Enterodiol</td>
<td>Phyto-oestrogen important for heart and bone health, and possible colon cancer protectants</td>
<td>Davis 2009</td>
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<td></td>
<td>Enteralactone</td>
<td>Phyto-oestrogen important for heart and bone health, and possible colon cancer protectants</td>
<td></td>
</tr>
</tbody>
</table>
Possible solution

A more holistic approach
Functional analysis of faecal water

• Functional analysis of faecal water provides an integrated measure of the overall contribution of the compounds present to a defined biological endpoint.

• Provides no information on compounds responsible for the functional effect
## Available assays

<table>
<thead>
<tr>
<th>Endpoint</th>
<th>Assay</th>
</tr>
</thead>
<tbody>
<tr>
<td>Genotoxicity</td>
<td>Bacterial mutagenicity</td>
</tr>
<tr>
<td></td>
<td>Comet assay in mammalian cells</td>
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<tr>
<td>Cell toxicity</td>
<td>Cytotoxicity</td>
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<td></td>
<td>Barrier function</td>
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<td></td>
<td>Invasive potential</td>
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<td></td>
<td>Red cell lysis</td>
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<tr>
<td>Cell proliferation</td>
<td>Cell number</td>
</tr>
<tr>
<td></td>
<td>Cell cycle analysis</td>
</tr>
<tr>
<td>Immune modulation</td>
<td>Expression of inflammatory markers</td>
</tr>
<tr>
<td>Gene expression</td>
<td>AP-1, COX-2,</td>
</tr>
</tbody>
</table>

- Limitation: lack of standardisation in target cells and sample preparation
- Few studies applied the approach to evaluate prebiotic effects
Metabolomics

- simultaneous monitoring of changes in a wide range of metabolites
  - No need for an *a priori* hypothesis
- $^1$H-NMR, LC-MS, GC-MS
- matrix: feces, urine, plasma, tissue homogenates
Applicability of systems biology

- Metabolome signatures are analysed in the context of overall biochemistry of the tissue or sample
- “Biochemical connectivity”
- Enables to analyse the overall impact of the microbiota on host-biochemistry and -function
Future needs for functional analysis of the microbiota

- Quantified microbiota species (OTU) composition (16S rRNA based)
  - Available
- Quantified microbiota function profile (metagenome)
  - Available
- Quantified microbiota gene expression profile (metatranscriptome)
  - Emerging
- Quantified microbiota protein expression profile profile (metaproteome)
  - Emerging
- Quantified microbiota metabolite profiles (metametabolome)
  - Available
Conclusions

• No formal systematic reviews evaluating the physiological or toxicological properties of bacterial fermentation metabolites were found.

• End products of saccharolytic fermentation, SCFA, may have varying effects on colonic health, host physiology, lipoprotein metabolism and appetite.

• Comprehensive reviews and experimental studies indicated that protein fermentation metabolites (phenol, p-cresol, indole, ammonia), typically considered as harmful metabolites, occur at concentration ranges in the colon such that no toxic effects are expected either locally or following systemic absorption.
Conclusions

• There is insufficient data published to support the use of any individual bacterial metabolite as faecal biomarker of gut health.

• Way to go:
  • Profiling of metabolites in the context of overall tissue biochemistry
  • correlation of (multivariate) metabolome signatures with microbial, dietary and physiological data
  ⇨ evaluation of the overall impact of the microbiota on host health and gut function

• Current limitation
  • the bioinformatics integration and interpretation of the data
  • the lack of studies measuring metabolite fluxes in different body compartments or biofluids to provide an accurate picture of colonic metabolite nutrikinetics.
Thank you!

Acknowledgements
Prebiotic Task Force, ILSI Europe