Towards Microbial Fermentation Metabolites as Biomarkers for Health Benefits of Prebiotics

Kristin Verbeke

for working group on Microbial Metabolism and Fermentation

SUPPORTED BY THE ILSI EUROPE PREBIOTICS TASK FORCE
## Expert group members

<table>
<thead>
<tr>
<th>Name</th>
<th>Institution/Company</th>
<th>Country</th>
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<tbody>
<tr>
<td>Prof. Kristin Verbeke – <strong>Chair</strong></td>
<td>KU Leuven</td>
<td>BE</td>
</tr>
<tr>
<td>Dr. Annick Bernalier</td>
<td>INRA</td>
<td>FR</td>
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<tr>
<td>Prof. Alan Boobis</td>
<td>Imperial College London</td>
<td>UK</td>
</tr>
<tr>
<td>Prof. Christine Edwards</td>
<td>University of Glasgow</td>
<td>UK</td>
</tr>
<tr>
<td>Dr. Anne Franck</td>
<td>Cargill</td>
<td>BE</td>
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<tr>
<td>Prof. Michiel Kleerebezem</td>
<td>Wageningen University</td>
<td>NL</td>
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<tr>
<td>Dr. Gunhild Kozianowski</td>
<td>Südzucker/BENEIO Group</td>
<td>DE</td>
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<tr>
<td>Dr. Arjen Nauta</td>
<td>FrieslandCampina</td>
<td>NL</td>
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<tr>
<td>Prof. Jeroen Raes</td>
<td>KU Leuven</td>
<td>BE</td>
</tr>
<tr>
<td>Dr. Kieran Tuohy</td>
<td>Fondazione Edmund Mach</td>
<td>IT</td>
</tr>
<tr>
<td>Dr. Ric van Tol</td>
<td>Mead Johnson</td>
<td>NL</td>
</tr>
<tr>
<td>Dr. Alessandro Chiodini</td>
<td>ILSI Europe</td>
<td>BE</td>
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<tr>
<td>Dr. Tobias Recker</td>
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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.
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>
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<tr>
<td>• Lactate</td>
<td>• Indoles</td>
<td>Derivatives of</td>
<td></td>
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<tr>
<td>• Succinate</td>
<td>• Branched chain fatty acids</td>
<td>o Phenyl acetic acid</td>
<td></td>
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<tr>
<td>• Alcohols</td>
<td></td>
<td>o Phenylpropionic acid</td>
<td></td>
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<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>
</tr>
<tr>
<td>o Carbon dioxide</td>
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</tbody>
</table>

- Hydrogen Sulphide
- Lactate
- Propionate
- Succinate
- Butyrate
- Ammonia
- Acetate
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- Phenyl acetic acid
- Phenylpropionic acid
- Mandelic acids
- Cinnamic acids
- Equols
- Hydroxy fatty acids
- Secondary bile acids
- Long chain aldehydes
Metabolism of SCFA

Absorption

90-95%

Colonocytes

acetate: 40-75%
propionate: 75-90%
butyrate: 90-98%

Splanchnic extraction

Liver

Systemic circulation

Urine

Extravascular organs

• 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

References:
Fernando et al. Benef Microbes 2010, 1, 197-207
McOrist et al. J Nutr 2011, 141, 883-889
Nemoto et al. Dig Dis Scie 2012, 57, 2955-2964
Schwiertz et al. Obesity 2010, 18, 190-195
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 Schäfer², Silvia Beijer³, Nicolaas A. Bos³, Christiane Donus⁴ and Philip D. Hardt⁴

Increased faecal SCFA excretion due to decreased SCFA uptake?

- MCT transporters expression and apical location is promoted by luminal SCFA

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

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

Yang et al. J Med Food 2013, 16, 862-867
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>
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</table>
| Ammonia    | • Bacterial degradation of amino acids  
|            | • Hydrolysis of urea |
| Phenols    | • Major metabolites of bacterial fermentation of aromatic amino acids  
|            | • Rapidly absorbed by colonic mucosa and excreted in urine  
|            | • Do not accumulate in healthy subjects |
|            | • p-cresol < tyrosine  
|            | • phenol < phenylalanine  
|            | • indole < tryptophan |
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

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. 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
Urinary and faecal levels of phenol

Damen et al. J Nutr 2012, 142, 470-477
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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 $\Rightarrow$ uremic toxin
  $\Rightarrow$ contributes to endothelial disfunction

- Very limited data on toxicity of indol
### List of microbial catabolites of common plant polyphenols and their putative health effects.

<table>
<thead>
<tr>
<th>Plant polyphenol</th>
<th>Microbial Catabolites</th>
<th>Possible health effects</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>(+)-epicatechin</td>
<td>4-hydroxyphenylacetic acid</td>
<td>Antimicrobial/antimycotic activity in vitro</td>
<td>Alakomi 2007[33], Ko 2009, Roowi 2010[34]</td>
</tr>
<tr>
<td></td>
<td>3-3-hydroxyphenyl(propionic acid)</td>
<td>Antimicrobial activity against Gram negative enterobacteria via outer membrane destabilization</td>
<td></td>
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<tr>
<td></td>
<td>5-3,4-dihydroxyphenyl-gamma-valeric acid</td>
<td>?</td>
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<tr>
<td></td>
<td>(-)-5-7,4' hydroxyphenyl-gamma-valerolactone</td>
<td>?</td>
<td></td>
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<tr>
<td>(-)-epigallocatechin</td>
<td>4-hydroxyphenylacetic acid</td>
<td>Antimicrobial/antimycotic activity in vitro</td>
<td>Roowi 2010[35]</td>
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<td>3-3-hydroxyphenyl(propionic acid)</td>
<td>?</td>
<td></td>
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<tr>
<td>(-)-epigallocatechin-3-O-glucoside</td>
<td>pyrocatechol</td>
<td>Antimicrobial/antimycotic activity in vitro</td>
<td></td>
</tr>
<tr>
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<td>Daidzein</td>
<td>Equol</td>
<td>Phytoestrogen important for heart and bone health, and possible colon cancer protectants</td>
<td>Jackman 2002[36], Ishimi 2009[37], Davis 2009[38], Salma 2009</td>
</tr>
<tr>
<td>Quercetin</td>
<td>O-demethylangolensin</td>
<td>estrogenic and/or antiestrogenic activity</td>
<td>Larrosa 2006[39], Salma 2009</td>
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<tr>
<td></td>
<td>2-3,4-dihydroxyphenylacetic acid</td>
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<td>3,4-dihydroxybenzoic acid</td>
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<td></td>
<td>Pterocarpin</td>
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<tr>
<td>Kaempferol</td>
<td>3,4-dihydroxyphenylphenylpropionic acid</td>
<td>Antimicrobial activity against Gram negative enterobacteria via outer membrane destabilization</td>
<td>Salma 2009</td>
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<tr>
<td>Naringenin</td>
<td>3,4-dihydroxyphenylphenylpropionic acid</td>
<td>Antimicrobial activity against Gram negative enterobacteria via outer membrane destabilization</td>
<td>Salma 2009</td>
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<td>(-)-epicatechin</td>
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<td></td>
</tr>
<tr>
<td>(-)-epigallocatechin-3-O-glucoside</td>
<td>pyrogallol</td>
<td>Anti-bacterial activity (especially against Gram negative enterobacteria),</td>
<td>Ko 2009, Roowi 2010[35], Okello 2012[37], Ni 2008, Taguri 2006[38]</td>
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<tr>
<td>Isoxanthohumol</td>
<td>8-prenylangolensin</td>
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<tr>
<td>Catechin and epicatechin</td>
<td>3,3-hydroxyphenyl(propionic acid)</td>
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<td></td>
<td>5-3,4-dihydroxyphenyl-gamma-valerolactone</td>
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<td>Ellagitannins/Ellagic acid</td>
<td>Urolithin-A</td>
<td>estrogenic and antiestrogenic activity, antimalarials</td>
<td>Del Río 2010[31], Larrosa 2006[40], Dell’Agli 2010[32]</td>
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<td>Urolithin-B</td>
<td>estrogenic and antiestrogenic activity, antimalarials</td>
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<td>Urolithin-C</td>
<td>estrogenic and antiestrogenic activity, antimalarials</td>
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<td></td>
<td>Urolithin-D</td>
<td>estrogenic and antiestrogenic activity, antimalarials</td>
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<td>Rutin</td>
<td>3,4-dihydroxyphenylacetic acid</td>
<td>Ruin and catabolites inhibit AGE formation in vitro; Antimicrobial activity against Gram negative enterobacteria via outer membrane destabilization</td>
<td>Alakomi 2007[33], Jaganath 2009[37], Cervantes-Laurean 2005[38]</td>
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</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.
<table>
<thead>
<tr>
<th>Endpoint</th>
<th>Assay</th>
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<tbody>
<tr>
<td>Genotoxicity</td>
<td>Bacterial mutagenicity</td>
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<td>Comet assay in mammalian cells</td>
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<tr>
<td>Cell toxicity</td>
<td>Cytotoxicity</td>
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<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>
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<td></td>
<td>Cell cycle analysis</td>
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<tr>
<td>Immune modulation</td>
<td>Expression of inflammatory markers</td>
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<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)
- Quantified microbiota function profile (metagenome)
- Quantified microbiota gene expression profile (metatranscriptome)
- Quantified microbiota protein expression profile (metaproteome)
- Quantified microbiota metabolite profiles (metametabolome)

Availability of methods:
- ✓
- ✓
- emerging
- emerging
- ✓
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