# Bacterial Impact on the Gut Metabolome

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# Bacterial Impact on the Gut Metabolome


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**DTU Food**
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# BACTERIAL IMPACT ON THE GUT METABOLOME

PH.D. THESIS BY KAROLINA SULEK


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DTU FOOD

TECHNICAL UNIVERSITY OF DENMARK

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# Preface

This thesis concludes research carried out mainly at the DTU Food (National Food Institute), Technical University of Denmark (DTU) stretching from 2009 until 2012. The studies were supervised by Professor Tine Rask Licht, research manager of the Intestinal Microbial Ecology group and co-supervised by Doctor Andrea Wilcks, Associated Professor Thomas Hjort Skov and Doctor Jørn Smedsgaard, head of Food Chemistry.

The work presented here is the result of a multidisciplinary project, aiming to study metabolic influence of the probiotic and prebiotics on the human health. Collaboration between the Division of Food Microbiology, Division of Food Chemistry at DTU, Copenhagen University and studies done at the Auckland University made the journey of gut ecology metabolomics feasible.

Project “Bacterial Impact on the Gut Metabolome” was supported by the Danish Strategic Research Council’s Committee on Food and Health, FøSu, Center for Biological Production of Dietary Fibers and Prebiotics; Øresund Food Network and Technical University of Denmark.

# Acknowledgements

Numerous people have interacted with me during the study. Let me start by recognizing Professor Tine Rask Licht and Doctor Andrea Wilcks for sharing the ungrateful job as Ph.D. supervisors throughout the project. I thank them for the many good and fruitful discussions we had along the way. I greatly appreciate their support in the cozy beginning, chaotic middle and crazy end of my Ph.D. studies.

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Professor Anne S. Meyer and Doctor Jesper Holck from the Technical University of Denmark are thanked for their prebiotic support.

Senior Lecturer, Doctor Silas Granato Villas-Boas from the Auckland University is thanked for giving me the opportunity to undertake my external research in his group, the Metabolomics Applied to Industrial and Medical Microbiology (MAIMM). I enjoyed my time there from the scientific point of view as well as the social aspect thanks to all of the MAIMM group members. I would also give special recognition to Farhana Rahman Pinu, Michael Yap and Doctor Dang-Dung Nguyen from MAIMM for a great deal of help during my visit there.

I am very grateful to all of my current and former colleagues from the Intestinal Microbial Ecology group for their support. My work would not be possible without the great technical support from Kate Vibefeldt and Bodil Madsen. For numerous stimulating talks and “hyggelig” time I would like to thank people from Office 007, especially during the freaking-out periods! I am also grateful for the everyday smiles from Division of Food Microbiology staff members.

I would like to dedicate this work to my family and friends all around the world. This would not be possible without the support of my beloved parents Wiesław and

Bożena Sułek and my awesome brother Ireneusz Paweł Sułek. Thank you for a great deal of moral support, your love and understanding.

(Wiem, że to niewiele wzamian, jednakże chciałabym zadedykowac tą pracę mojej rodzinie i przyjaciołom rozsianym po całym świecie. Praca ta nie byłaby możliwa bez wsparcia ze strony moich ukochanych rodziców Wiesława i Bożeny Sułek oraz mojego genialnego braciszka Ireneusza Pawła Sułek. Dziekuję Wam za ogromne moralne wsparcie, Waszą miłość i zrozumienie.)

To my voluntary reviewers Beata Renata Jonach and Casper N. Søgaard - thank you for your precious time.

Last, but defiantly not least big gratitude goes to the love of my life, Michel Fruergaard Masana. Thank you for bearing with me throughout the whole project, being with me in both the sweet and very hard times, for your patience and for the way you are.

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Karolina Sułek

# Summary

The human gastrointestinal tract (GIT) is colonized by a dense and complex community of bacteria. This affects the host through modulation of the immune system, protection against pathogens, development of the intestinal microvilli, enteric nerve regulation, promoting angiogenesis and conversion of nutrients and metabolites. Therefore, human global metabolism at the whole-body level is the integration between the activities of our genome and the microbiome. As the human GIT provides nutrients to cells and tissues via the circulatory system, so do the metabolic products from the microbial flora. In other words, every human cell is somehow influenced by metabolites originating from the gut microbiota. This cross-talk between the microbes and the host includes signaling via low molecular weight metabolites, peptides and proteins. Throughout the decades of studies it has become evident that the intestinal microbiota can be modulated by intake of probiotic and prebiotic dietary supplements. Recently scientists have addressed the effects of these dietary interventions on the presence of specific bacterial metabolites, which are anticipated to play a role in gut health.

In this thesis, by using the metabolomics tools, the impact of the selected well-described probiotic bacteria, *Lactobacillus acidophilus* NCFM, on the simplified intestinal metabolome of the germ-free animals was evaluated. The studies attempted to map metabolites produced by the NCFM strain when growing *in vitro,* and in the intestinal environment as well as mapping host metabolites induced by the presence of the bacteria. *In vitro* studies with NCFM showed that this strain in the presence of a simple carbohydrate source (glucose) increased the concentration of lactic acid, succinic acid, adenine and arginine in the medium after 24 h of fermentation, using adenosine and glucose as the primary source of energy. Lactic acid and succinic acid, produced by the probiotic strain could have a beneficial effect on the host, lowering the pH in the intestines and thereby protecting from pathogenic infections and cancer development. However, the GIT is a much more complex environment that is affected by the nutrition available for the bacterial fermentation and mammalian metabolites interacting with the probiotic. Therefore, a simplified mammalian model, the germ-free mice studies, was established to analyze the influence of NCFM on the host. Our initial analyses, comparing germ-free and

monocolonized animals with NCFM, showed a distinctive differences in the metabolism throughout the mammalian GIT as well as global metabolism, represented by plasma and liver samples.

To increase the knowledge about bacterial metabolites created by prebiotic fermentation, and the impact of the size (chain length) of the sugar beet arabino-oligosaccharides (AOS), *in vitro* fermentations with human fecal microbial communities were used. The metabolic and phylogenetic response to high-mass AOS was found to be the most similar to commercially available prebiotic fructo-oligosaccharides. High-mass AOS in comparison to the low-mass and base fraction (mixture of the two fractions) caused the highest increase of metabolites putatively beneficial to the human gastrointestinal tract.

Probiotic and prebiotic influence on the host looks for the beneficial aspect of the bacterial flora. However the GIT is not only inhabited by beneficial microbiota, but also potential pathogens. A balanced microflora requires that the bacterial composition work in a co-metabolic symbiotic relationship with the host, supporting the complex system. An unbalanced composition might potentially be the beginning of many diseases, such as inflammatory bowel diseases and, in particular, Ulcerative Colitis (UC). Our metabolomic studies have shown significant differences in the metabolism between microflora from UC patients in relapse and remission or healthy individuals. The metabolomic-angled look on UC microflora constitutes an important contribution to the understanding of the complex etiology behind UC.

In conclusion, studies of the GIT bacterial activity revealed a potentially significant impact of the gut microflora and on the host metabolome in relation to the dietary modulation and in the diseased state of UC.

# Dansk sammendrag

Den menneskelige mave-tarmkanal er koloniseret af en tæt og meget kompleks gruppe af bakterier. Dette påvirker værten gennem modulation af immunforsvaret, beskyttelse mod patogener, udvikling af mikrovilli i tarmen, regulering af enteriske nerver, fremning af blodkardannelse og omdannelse af næringsstoffer og metabolitter. Det globale menneskelige stofskifte for hele kroppen er derfor integrationen mellem aktiviteterne i vores genom og aktiviteterne i det mikrobiologiske biom. På samme måde som menneskets mave-tarmkanal leverer næringsstoffer til celler og væv via blodomløbet, ligeledes leveres metabolitter fra den mikrobiologiske flora. Med andre ord, hver eneste menneskelige celle er påvirket af metabolitter der stammer fra de mikrobiolgiske økosystem i mave-tarm systemet. Denne tværgående kommunikation mellem mikrober og værten inkluderer signalering via lav-molekylærvægt metabolitter, peptider og proteiner. Gennem flere årtiers studier er det blevet tydeligt at de mikrobiolgiske bakteriefamilier kan moduleres ved indtagelse af pro- og prebiotiske kosttilskud. I senere tid har forskere undersøgt hvilke effekter denne kost-forårsagede indgriben har på tilstedeværelsen af specifikke bakterielle metabolitter, som er forventet at spille en rolle i mave-tarm systemets helbred.

I denne afhandling blev indvirkningen af den udvalgte, velkendte probiotiske bakterie *Lactobacillus acidophilus* NCFM på det forsimplede mave-tarm metabolom i kimfri dyr evalueret, ved hjælp af metabolomics teknikker. Studierne forsøgte at kortlægge metabolliter der blev produceret af NCFM stammen, når den voksede henholdsvis *in vitro* og i mave-tarm miljøet og ydermere at kortlægge værts metabolitter induceret af bakteriens tilstædeverelse. *In vitro* studier med NCFM viste at denne stamme, efter 24 timers fermentering, øgede koncentrationen af mælkesyre, ravsyre, adenin og arginin i mediet i tilstædeværelsen af en simpel kulhydratkilde (glukose). Glukose og adenosin var de primære energikilder. Mælke- og ravsyren produceret af den probiotiske stamme kunne havde en gavnlig effekt på værten ved at sænke pH-værdien i mave-tarm systemet og derved beskytte mod patogen infektioner og udvikling af kræft. Den menneskelige mave-tarmkanal er dog et mere kompleks miljø der bliver påvirket af næringen brugt i bakteriel fermentering og mammale metabolitter der interagerer med probioten. Derfor blev en

forsimplet mammal model, studier i kimfri mus, etableret for at analyserer hvilken indflydelse NCFM har på værten. Vores indledende analyser, der sammenligner kimfri dyr med dyr enkeltkoloniserede af NCFM, viste en tydelig forskel i metabolismen i den mammale mave-tarmkanal såvel som i den globale metabolisme. Dette blev påvist ved hjælp af prøver af blodplasma og lever.

For at øge viden om bakterielle metabolitter skabt ved prebiotisk fermentering og indflydelsen af størrelse (kædelængden) af arabino-oligosakkarider (AOS) fra sukkerroer, blev *in vitro* fermentering med mikrobiologiske kulturer fra menneske fækalier. Det metaboliske og fylogenetiske respons på høj-masse AOS blev bedømt til at være den samme respons som set med kommercielle prebiotiske frukto-oligosakkarider. Høj-masse AOS gav i forhold til lav-masse og basis fraktioner (en blanding af de to fraktioner) den højeste stigning af metabolitter, der er formodet gavnlige for den menneskelige mave-tarmkanal.

Pro- og prebiotisk indflydelse på værten ser på det gavnlige aspekt af den bakterielle flora. Men mave-tarmkanalen er ikke kun beboet af gavnlige mikrober, den indeholder også potentielle patogener. En balanceret mikrobiologisk flora kræver, at den bakterielle sammensætning arbejder sammen i symbiotisk metabolisk samarbejde med værten, der støtter det komplekse system. En ubalanceret sammensætning kan potentielt være begyndelsen af mange forskellige sygdomme, som f.eks. inflammatorisk mave-tarm sygdomme og, især, Ulcerativ Colitis (UC). Vores metabolomiske undersøgelser har vist tydelige forskelle i metabolismen mellem den mikrobiologiske flora i UC patienter, der er har henholdsvis tilbagefald, aftagene symptomer og i raske individer. Den metabolomics-baserede indgangsvinkel på UC mikrobiologisk flora udgør en vigtig tilføjelse til forståelsen af den komplekse etiologi bag UC.

For at konkludere, undersøgelser af den mave-tarmkanal bakterielle aktivitet afslørede en potentiel tydelig effekt på mave-tarm systemets mikrobiologiske flora og værtens metabolom, i forhold til diætmodulation og sygdomstilstanden fra UC.

# Objectives of the study

During the last decade, it has become evident that the complex ecosystem of microbes inhabiting the human gut plays an important role for human health [1-3]. Additionally, it has become evident that the intestinal microbiota can be modulated by intake of probiotic and prebiotic dietary supplements [4,5]. A large number of studies have addressed the effects of dietary interventions on the presence of specific bacterial metabolites, which are anticipated to play a role for gut health [6]. Recently developed approaches allow simultaneous mapping of multiple metabolites present in the gut metabolome [6-13].

By using the metabolomics tools, the impact of selected well-described probiotic bacteria, *Lactobacillus acidophilus* NCFM, on the simplified intestinal metabolome of the germ-free animals was evaluated. This project attempted to map metabolites produced by the NCFM strain when growing *in vitro,* and in the intestinal environment as well as host metabolites induced by the presence of the bacteria (Manuscript I and II).

Non-digestible carbohydrates are mainly known for the influence on the short chain fatty acids production. To increase the knowledge about bacterial metabolites created by prebiotic fermentation, and the impact of the size (chain length) of the sugar beet arabino-oligosaccharides, *in vitro* fermentations with human fecal microbial communities were used (Manuscript III).

Additionally, in search for the etiology of the Ulcerative Colitis, metabolome differences between fecal microbial communities of patients and healthy subjects were studied. Bacterial influence on the gut was taken into consideration as one of the source of the disease (Manuscript IV).

As bacterial impact on the gut is still a big puzzle, the project aimed at examining the metabolic mechanisms between host and microbiota in order to make this puzzle more solvable.

# List of publications

### Paper published

- **Sulek, K.**; Frandsen, H.L.; Smedsgaard, J.; Skov, T.H.; Wilcks, A.; Licht, T.R. (2012); Metabolic footprint of *Lactobacillus acidophilus* NCFM at different pH; *Metabolomics*, 8, 2:244-252; DOI: 10.1007/s11306-011-0305-4 (2011).

### Data in preparation

- **Sulek, K.**; Skov, K.; Frandsen, H.L.; Smedsgaard, J.; Skov, T.H.; Wilcks, A.; Villas-Boas, S.G.; Licht, T.R.; *Lactobacillus acidophilus* NCFM effect on the host metabolome in the monocolonisation studies of the germ-free mice.

### Manuscripts

- **Sulek, K.**; Vigsnaes, L.K.; Schmidt, L.R.; Holck, J.; Frandsen, H.L.; Smedsgaard, J.; Skov, T.H.; Meyer, A.S.; Licht, T.R. (2012); A combined metabolomic and phylogenetic study reveals putatively prebiotic effects of high molecular weight arabino-oligosaccharides when assessed by in vitro fermentation in bacterial communities derived from humans.
  *(Manuscript I)*

- Vigsnaes, L.K. and van den Abbeele, P.; **Sulek, K.**; Frandsen, H.L.; Steenholdt, C.; Brynskov, J.; van de Wiele, T.; Licht, T.R. (2012); Microbiotas from UC patients have an altered metabolism and a reduced ability of lactic acid bacteria to colonize mucus.
  *(Manuscript II)*

**Poster presentations** (abstracts not included in this thesis)

- **Sulek, K.**; Vigsnaes, L.K. and van den Abbeele, P.; Frandsen, H.L.; Steenholdt, C.; Brynskov, J.; van de Wiele, T.; Licht, T.R. (2012); Metabolic footprint of UC bacterial ecosystem compared to the healthy donors; 7. Danish Conference on Biotechnology and Molecular Biology, Vejle, Denmark.

- **Sulek, K.**; Vigsnaes, L.K and van den Abbeele, P.; Frandsen, H.L.; Steenholdt, C.; Brynskov, J.; van de Wiele, T.; Licht, T.R.; Metabolic footprint of UC bacterial ecosystem compared to the healthy donors; Metabolomics 2012, Washington DC, USA.

- **Sulek, K.**; Frandsen, H.L.; Smedsgaard, J.; Skov, T.H.; Wilcks, A.; Licht, T.R.; Metabolic footprint of *Lactobacillus acidophilus* NCFM at different pH; Metabolomics 2011, Cairns, Australia.

- **Sulek, K.**; Frandsen, H.L.; Smedsgaard, J.; Skov, T.H.; Wilcks, A.; Licht, T.R. (2010); The effect of different in vitro conditions on the metabolic footprint of *Lactobacillus acidophilus* NCFM; International Scientific Conference on Probiotics and Prebiotics, 2010, Kosice, Slovakia.

- **Sulek, K.**; Frandsen, H.L.; Smedsgaard, J.; Skov, T.H.; Wilcks, A.; Licht, T.R. (2010); Bacterial Impact on the Gut Metabolome; Metabolomics and More. The Impact of Metabolomics on the Life Sciences, 2010, Freising, Germany.

# Abbreviations

| | |
|---|---|
| **AOS** | Arabino-oligosaccharides |
| **BA** | Bile acids |
| **BSH** | Bile salt hydrolase |
| **CA** | Cholic acid |
| **CE** | Capillary electrophoresis |
| **CFU** | Colony forming unit |
| **DI** | Direct infusion |
| **DP** | Degree of polymerization |
| **FDA** | Fisher discriminant analysis |
| **FOS** | fructo-oligosaccharides |
| **FT-IR** | Fourier transform-infrared |
| **GABA** | γ-aminobutyric acid |
| **GC** | Gas chromatography |
| **GIT** | Gastrointestinal tract |
| **GOS** | Galacto-oligosaccharides |
| **GPC** | Glycerophosphorylcholine |
| **GSH** | Glutatione |
| **HBF** | Human baby flora |
| **HDL** | High-density lipoproteins |

| | |
|---|---|
| **HSDH** | Hydroxysteroid dehydrogenase |
| **LC** | Liquid chromatography |
| **LDL** | Low-density lipoprotein |
| **MS** | Mass spectrometry |
| **M-SHIME** | Simulator of the Human Intestinal Microbial Ecosystem with incorporated mucin-covered microcosms |
| **NMR** | Magnetic resonance spectroscopy |
| **PC** | Principal component |
| **PCA** | Principal component analysis |
| **PLS-DA** | Partial least squares discriminant analysis |
| **SCFA** | Short-chain fatty acid |
| **TCA** | Taurocholic acid |
| **TOF** | Time of flight |
| **TβMCA** | Tauro- β-muricholic acid |
| **UC** | Ulcerative Colitis |
| **VLDL** | Very-low-density lipoprotein |
| **βMCA** | β-muricholic acid |

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# Bacterial Impact on the Gut Metabolome

## Theoretical part

# 1. Metabolomics

## 1.1. Metabolomics concept

Metabolites are classified as low-molecular-weight compounds, which are related to cell metabolism, mostly described as chemicals below 1 kDa [6]. As intermediates of biochemical reactions, metabolites have a very important role in connecting many different pathways [14] in and across organisms. The complete set of metabolites associated with an organism is referred to as a metabolome, which is divided into endo- and exo-metabolomes, covering intra- and extra-cellular metabolites, respectively. Metabolomics is a research field which aims at comprehensive analysis of a metabolome. An unspecific analysis of intracellular metabolites is called **metabolic fingerprinting**. Metabolomics focused on the untargeted extracellular part of the metabolome is designated as **metabolic footprinting** [15].

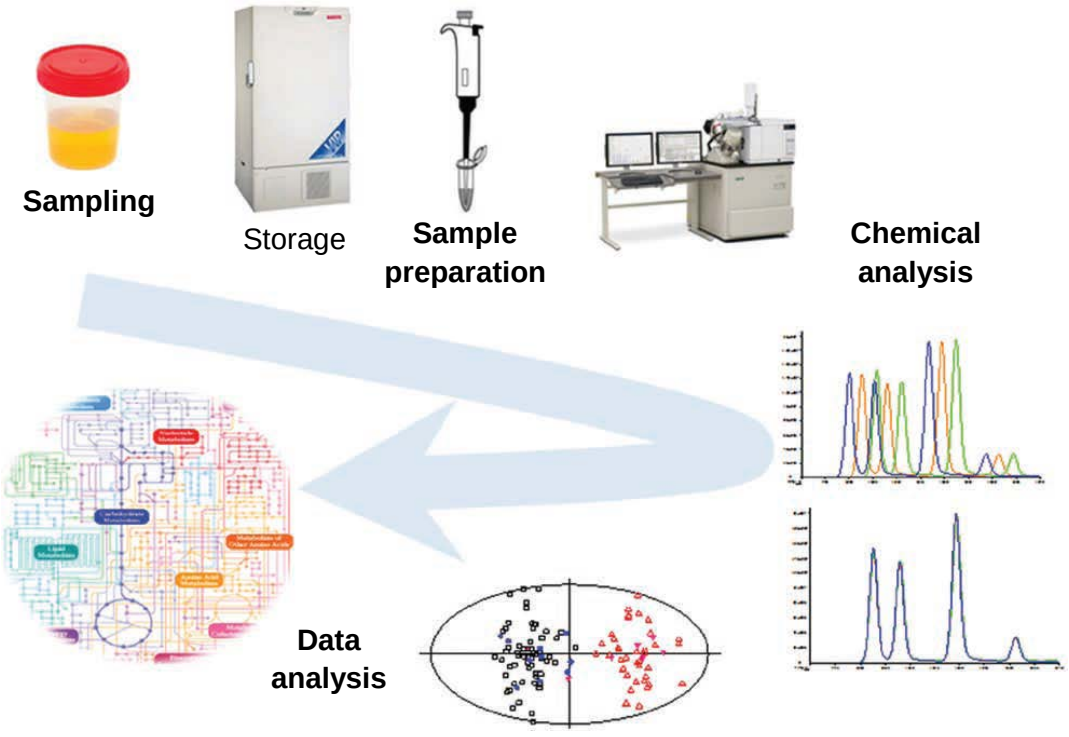


**Figure 1.1 A flow chart of a metabolomic study. As an example the metabolic profiling of urine samples is shown. Figure modified from Chan *et. al,* 2011 [16].**

Metabolomics involves sampling, sample preparation, chemical analysis and data analysis. A flow chart of a “standard” metabolomic study is shown in Figure 1.1.

## 1.2. Metabolic footprinting

In order to analyze the bacterial impact on the gut metabolome this thesis is focused on metabolic footprinting, a set of non-targeted biochemistry studies and data mining of extracellular metabolites [17] obtained from the *in vitro* growth cultures or body fluids (milk, plasma, urine). Despite the fact that footprinting represents only a small fraction of the whole metabolome, it provides a key understanding of cell and organism communication mechanisms, which play a crucial role in the symbiotic relationships between gastrointestinal tract (GIT) microflora and the host. In an extracellular environment any changes in the abundance and level of extracellular metabolites will directly reflect any modifications of the environment caused by activities of microorganism present in the system. The connection between the microbial activity (intracellular metabolome) and excreted metabolites is presented in Figure 1.2 [18].

The microbial metabolism is directly influenced by the external environment. Metabolites released into the environment are a consequence of cell metabolic activity, modifying the external environment. Therefore analysis of the metabolic footprint provides an insight into possible microbial metabolic activities that accrues in relation to the presence of natural products and potential xenobiotics.

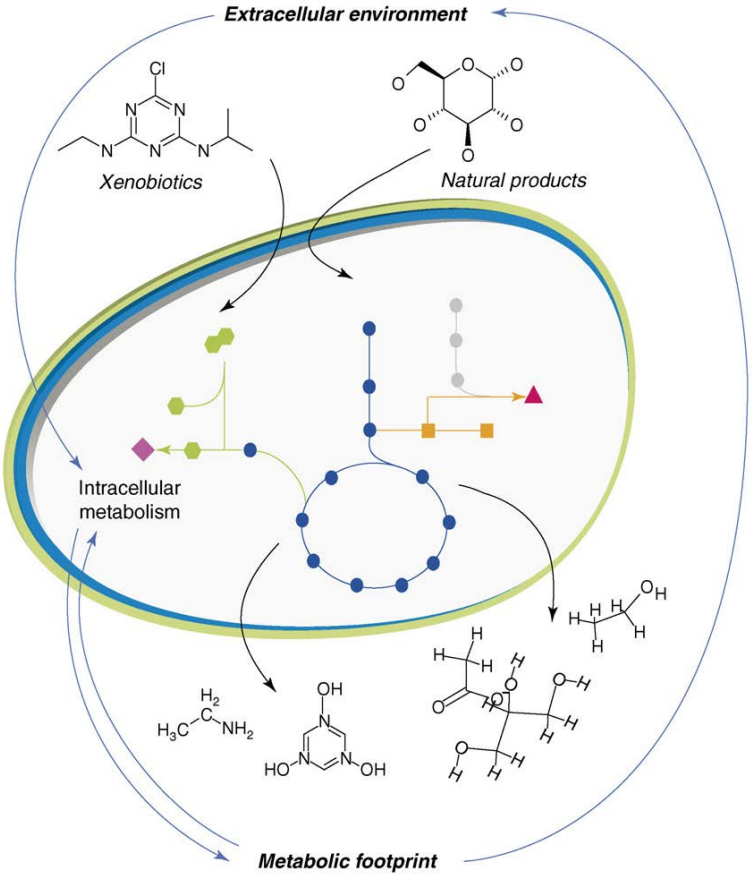


**Figure 1.2 Coupling between the environment and cell metabolism. Metabolites shown in the figure serve as examples. Figure from Mapelli *et. al,* 2008 [18].**

## 1.3. Metabolomic tools

As previously mentioned metabolomics aims at the comprehensive analysis of a metabolome. Choosing a suitable analytical strategy require several considerations: the information needed, the chemistry expected and the analytical facilities available. The nature of the metabolome, intracellular and extracellular, is very complex and no single methodology can detect the complete metabolome in one procedure. Key parameters for this choice are given in Table 1.1.

### 1.3.1. Sampling and sample preparation

The first and most crucial steps in metabolomic studies are sampling and sample preparation. The methodology of sampling and sample preparation for fingerprinting of metabolites is dependent on the type of sample (bacterial, yeast cells, animal or plant

**Table 1.1 Key parameters to take into consideration when choosing analytical methods. Table adapted from Villas-Boas *et. al*, 2007 [6].**

| | |
|---|---|
| Physicochemical properties of metabolites | Molecular weight and size<br>Polarity (polar, non-polar)<br>p$K_a$ (acidic, alkaline, neutral)<br>Concentration (sensibility of detectors)<br>Detectability (chromophors, ionizability, etc.)<br>Volatility |
| Concentration | Trace or massive amount |
| Matrix | Interference from co-extracted substrate or from major components in the sample |

tissue). Comprehensive information, about the choice of methods for metabolism quenching and intracellular metabolite extraction is presented by Villas-Boas *et al.* [19]. General steps involved in sample preparation of extracellular metabolites are presented in Figure 1.3.

Cellular metabolism is a dynamic process with very different turnover speeds, depending on the concentration of enzymes, substrate availability etc. Analysis of the extracellular metabolites requires a fast separation from the cells, which is usually achieved by cold centrifugation (1-4°C). Low temperature during centrifugation is necessary to slow down the cellular metabolism. Extracellular metabolite turnover, compared to intracellular metabolite, is much slower, due to the higher dilution of metabolites. However due to several issues, including the presence of cell lyses, release of the intracellular matrix, changes in the substrate concentration, activity of extracellular enzymes, chemical degradation and chemical interactions, metabolic footprinting still requires a rapid inactivation of the metabolism; the metabolism quenching. Inactivation of chemical and enzymatic activities is usually done by placing the biological sample in contact with cold (< -40°C) or hot (> 80°C) organic solutions or by dramatically changing the pH, typically by addition of perchloric acid. Use of liquid nitrogen is also possible, but not very common. Storage below -20°C before the analysis prevents further degradation of the samples.

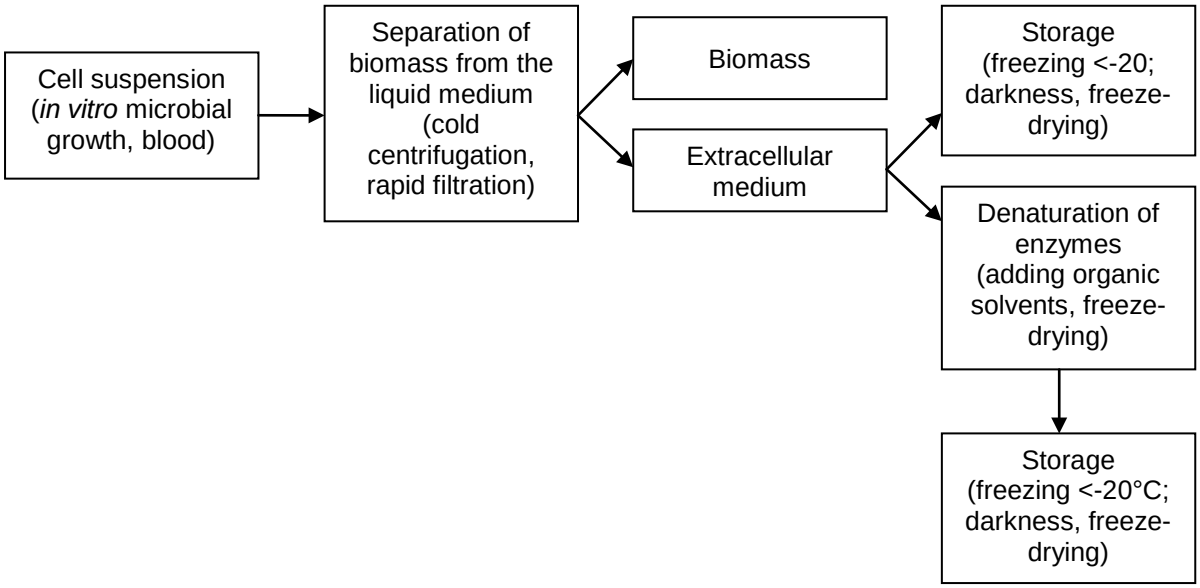


**Figure 1.3 Sampling and sample preparation of extracellular metabolites. Figure modified from Villas-Boas *et. al*, 2007 [6].**

### 1.3.2. Chemical analysis

A wide range of analytical tools are used in metabolomics. Some of the more common methods are: vibrational spectroscopy, nuclear magnetic resonance (NMR) spectroscopy and mass spectrometry (MS).

#### Vibrational spectroscopy

Fourier transform-infrared (FT-IR) is a method used to measure the overall composition of a sample by detecting the molecular vibrations and other motions of chemical bonds [20]. It has the benefit of enabling rapid, reagentless, non-destructive analysis of very complex biological samples. The drawback of this method is its low sensitivity, making the results difficult to integrate with biological information [18].

#### Nuclear magnetic resonance (NMR)

Continuous improvements in resolution and sensitivity over the years, has made NMR one of the most used techniques in metabolomics [21-25]. The main benefit of NMR is that it is specific and nonselective, which means that each resonance observed is specific to a particular compound and provides structural information regarding the components of a sample. It does not need a pre-selection of analysis conditions, like MS systems [18].

Additional aspect of NMR is its non-invasive nature, which allows *in vivo* metabolome profiling [26].

**Mass spectrometry (MS)**

Many different and complementary mass spectrometry platforms are employed in metabolomic studies. The most recent and common platforms are: direct infusion mass spectrometry (DI-MS), gas chromatography, liquid chromatography and capillary electrophoresis coupled with MS (GC-MS, LC-MS and CE-MS, respectively).

With using the DIMS platform metabolites are injected directly in front of the ion source, bypassing any separation methods like LC or GC. For this type of analysis time of flight (TOF) mass spectrometers are the preferred instruments, providing additional separation of ions, depending on their molecular mass. Additionally for all types of MS, TOF spectrometers can provide full mass scan abilities and complete mass spectra with good sensitivity [27]. DI-MS also allows high-throughput analyses of biological samples. The major problem with DI-MS technology is the “matrix effect” [28] and even with the use of a TOF spectrometer, DI-MS does not provide a good enough separation of metabolites. The mentioned effect can also compromise sensitivity and accuracy of mass analysis [29]. Ion suppression, caused by coeluting compounds and isobaric interferences, are also a major disadvantage of DI-MS.

CE-MS, GC-MS and LC-MS give a good separation of metabolites, reducing problem related to the direct infusion and also provide the possibility for separation of isomers. A good analytical separation will also result in better detection limits and improve MS data quality [29].

CE-MS is a high-resolution technique. However for each type of metabolites (anionic, cationic, nucleotides, etc.) a separate methodology needs to be set up to achieve a comprehensive coverage of metabolome, which prolongs the analysis time per sample [30].

The most commonly used metabolomic techniques are GC-MS and LC-MS. The combination of gas chromatography with electron impact MS provides high chromatographic metabolite resolution, analyte-specific detection, quantification of

metabolites and the possibility to indentify unknown metabolites [31]. However, a major disadvantage of GC-MS is that analysis of polar metabolites usually requires derivatisation, increasing their thermal stability and volatility, which complicates the sample preparation process. The choice of reagents must be based on the stability of the derivatives and a low amount of by-products from the derivatisation process. Moreover, artifacts can be formed during derivatisation [32], complicating data interpretation. A big advantage of the GC-MS system is the presence of many metabolic databases, making the identification of componds much easier compared to the LC-MS [33].

Liquid chromatography gives a good separation of metabolites. Depending on the type of LC a wide range of metabolites can be covered by this method through the ionization in positive and negative mode with very good sensitivity. LC-MS does not require derivatisation, making the sample preparation easier to perform relative to GC-MS. LC-MS enables the possible analysis of thermo-labile metabolites. A few drawbacks of this system are possible matrix effects, sometimes requirement on desalting the samples, limited structural information and identification [34,35]. Identification difficulties could be overcome by the use of MS-MS techniques [36].

### 1.3.3. Data analysis

Chemical analysis of the metabolome results in a large number of data points per sample, placing the data in a multidimensional space. Ideally all measured metabolites should be identified. However, among the great number of variables often only a few are of interest, these few providing necessary biological information. The data processing in metabolomics aims at finding and identifying those few important variables. A flow chart of the metabolomic data analysis is shown in Figure 1.4.

First the acquired data is converted into a matrix as used by most of the multivariate analysis. Preprocessing of data requires signal to noise improvement and data normalization. Multivariate data analysis allows evaluation of the data quality, based on the replicates of samples and detection of outliers. More importantly, this greatly reduces the amount of metabolic candidates for further identification. A summary of the main multivariate methods and their use is given in Table 1.2 [37].

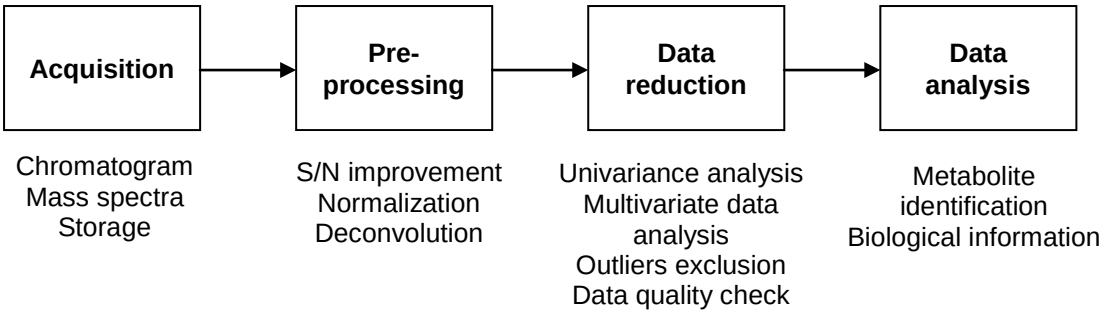


**Figure 1.4 Data analysis flow chart in relation to the metabolomic studies (S/N signal to noise ratio) [6,17,19].**

Extensive information about the methods in Table 2 can be found in Lattin *et al.*. In metabolomics the most popular methods reducing the dimension are Component Analysis (CA) and Discriminant Analysis (DA), often used as complimentary methods [24,38-40]; CA as an independent (unsupervised) method and DA as a dependent (supervised) method.

Principal Component Analysis (PCA) uses an orthogonal transformation to convert a set of observations, possibly correlated variables into a set of values, linearly uncorrelated variables called principal components (PC). This transformation is defined in such a way that the first principal component has the largest possible variance, which means that it accounts for as much of the variability in the data as possible. Each succeeding component in turn has the highest variance possible under the constraint that it be orthogonal to (uncorrelated with) the preceding components.

This way the PCs describing the highest number of variations can be selected to represent the data in a lower dimensional space. PCA results in the decomposition of raw data into “scores”, which reveal the relationship between samples and into “loadings” that show the relationships between the variables (Figure 1.5).

**Table 1.2 Examples of multivariate methods. Table adapted from Lattin *et. al*, 2003 [37].**

| Method | Type of Analysis | Objectives |
|---|---|---|
| Principal Components | **I, E** | Dimension reduction |
| Factor Analysis | **I, E** or **C** | **E** - Understand patterns of intercorrelation; uncover latent traits and **C** – verify measurements models |
| Multidimentional Scaling | **I,** mainly **E** | Create spatial representation from object similarities |
| Cluster Analysis | **I, E** | Create groupings from object similarities |
| Canonical Correlation | **D,** mainly **E** | Explain covariation between two sets of multiple variables |
| Structural Equation Models with Latent Variables | **D, C** | Dependence model with measurement error |
| Analysis of Variance | **D, C** | Special case of canonical correlation with discrete X variables |
| Discriminant Analysis | **D, E** or **C** | Special case of canonical correlation with discrete Y variables |
| Logit Choice Models | **D, E** or **C** | Nonlinear probability model for discrete choice outcomes |

**I**, **D**, **E** and **C** designated as **I**ndependent; **D**ependent; **E**xploratory and **C**onfirmatory analysis respectively

For the PCA method to be successful in pattern visualization it should capture the group segregation. However, PCA can be affected by noise in the variables (e.g. matrix in chromatogram analysis), which could distort the analysis. PCA, maximizing the variation, does not consider group information. Discriminant analysis, such as Fisher (linear) Discirminant Analysis (FDA) and Partial Least Squares Disciriminant Analysis (PLS-DA), maximizes between-groups variation, while minimizing the within-group variation. FDA and

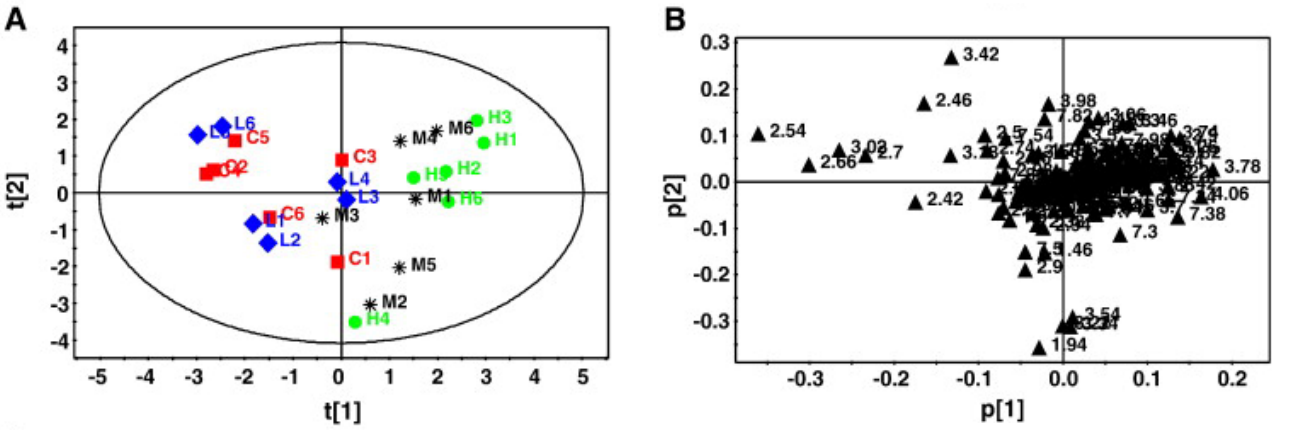


**Figure 1.5 Example of PCA plots; (A) PCA scores plot based on $^{1}$H NMR spectra of urine samples from rats; (B) PCA loading plot corresponding to the score plot. Figure from Ronghui *et. al*, 2008 [41].**

PLS-DA are supervised methods that use given grouping information for the data projection, which captures the group information and neglects the noise. Supervised methods have the tendency to overfit the data [37]. Therefore, cross-validation (technique for assessing how the results of a statistical analysis generalize to an independent data set) of the models is always necessary. There are many factors that may impact the result of statistical analysis of metabolomic data, such as choice of supervised and unsupervised methods, the normalization and scaling techniques. It is therefore wise to investigate several different options.

The last step in the metabolic studies (Figure 1.4) is the metabolite identification and correlation with the biological information. LCMS and GCMS systems often provide peak detection, identification and integration. Most software includes additional analytical tools, which are able to use either self-created or commercial mass spectra libraries for compound identification. However, GCMS provides more stable retention times between the systems, allowing system to system comparisons and presence of many commercially available databases [33]. Based on the exact mass to charge (m/z) ratio and MSMS analysis, LCMS online data bases [42,43] provide appropriate information allowing metabolite identification. Biological interpretation of the data is often based on the metabolic pathway correlations [14] publically available and standard literature studies, which connects the pieces of the metabolomic puzzle.

# 2. Human gastrointestinal tract

## 2.1. Human digestive system

The human GIT is divided into the oral cavity, esophagus, stomach (upper digestive tract), small intestine, colon, rectum and anus (large intestine). The small intestine is composed of the duodenum, jejunum and ileum. The GIT together with the associated organs, salivary glands, liver, gallbladder and pancreas constitute the digestive system (Figure 2.1). The digestive system is responsible for the breakdown and modification of food into smaller portions and usable nutrients, electrolytes, and fluids. In addition, it excretes unabsorbed residues, provides a protective barrier against the entry of toxic substances and infectious agents, serves as the largest endocrine organ in the body and interacts with other endocrine organs: the nervous system, circulatory system, immune system, and so on [44]. The digestion and absorption of nutrients depends on the softening, mixing and movement of the content along the GIT.

In the oral cavity, foods are masticated and lubricated with saliva. This process initiates the breakdown of food in a manner that will allow penetration and action of digestive enzymes. The secretion from the salivary glands contains enzymes; primarily α-amylase. The stomach is a capacious organ that mixes ingested food with gastric secretions to facilitate digestion. It is interposed between the esophagus and the small intestine. Digestive enzymes from pancreas and bile acids from the gallbladder mix in the duodenum. The digestive enzymes break down proteins and bile emulsify fats into micelles. The duodenum contains Brunner's glands that produce bicarbonate, which in combination with bicarbonate from pancreatic juice neutralizes stomach acids from the stomach. The jejunum, connecting the duodenum and the ileum as well as the colon, is able to absorb nutrients into the bloodstream due to a large surface area. Nutrients include monosaccharides, amino acids and fatty acids and water [45]. The time it takes a substance to travel through the entire GIT is on average between 24 and 72 hours, of which most (18-64 hours) is spent in the colon. Transit time through the stomach and small intestine is only about 4 to 8 hours [46].

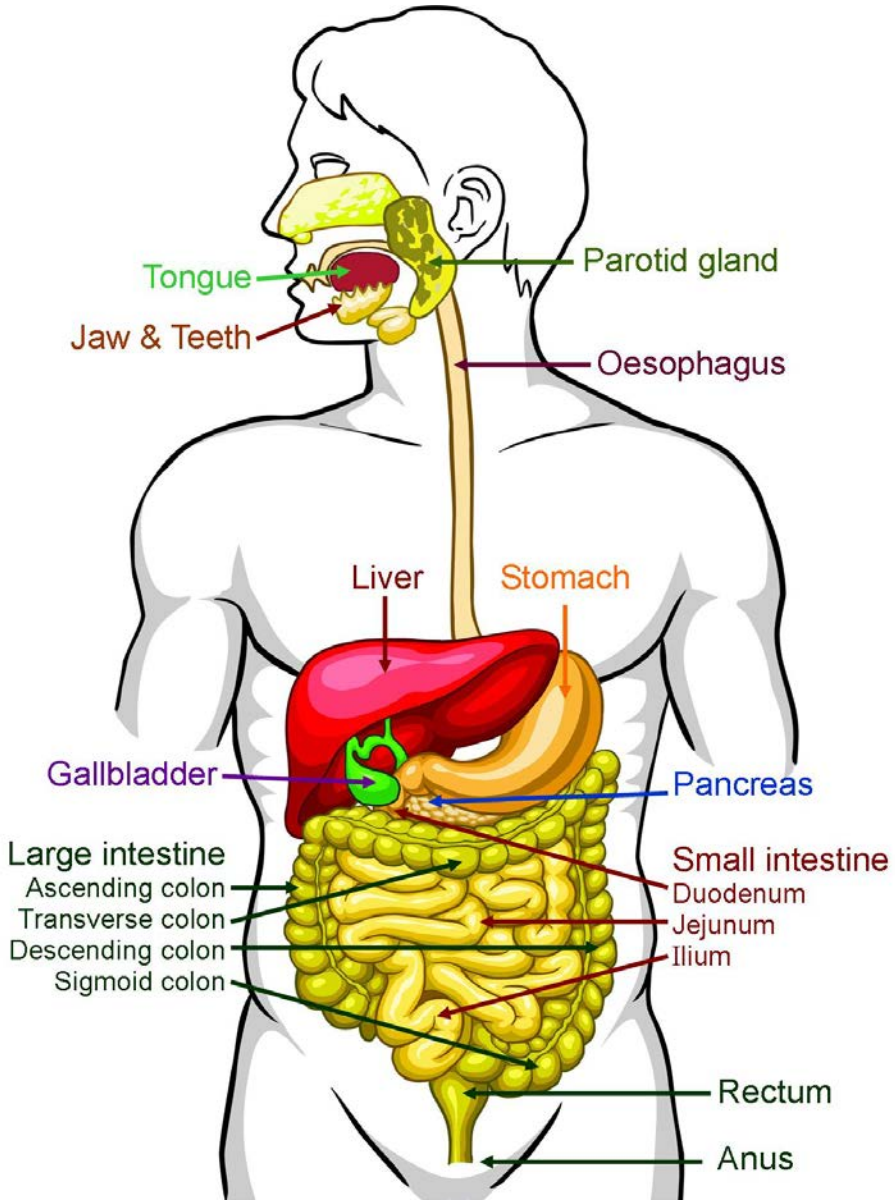


**Figure 2.1 Schematic representation of the human digestive system [47].**

## 2.2. Microbiota in the gastrointestinal tract

The human GIT is colonized by archaea, eukarya and a dense and complex community of bacteria all of which have a large impact on the health of the host. The host is affected through modulation of the immune system, protection against pathogens, development of the intestinal microvilli, enteric nerve regulation, promoting angiogenesis and conversion of nutrients and metabolites [48]. Proportionally, the human body consists of only 10% human cells and the remaining 90% are cells of microbial origin. Thus, there is more than

one genome within the human body, the human genome and the microbial, creating the symbiotic organism recently described as “superorganism” [49]. The total number of microorganisms in the GIT is around $10^{14}$ and varies greatly between different regions of the gut [50]. Despite the importance of the GIT microflora, little is known with respect to the microbial composition, species diversity and their metabolic activity. However, recently developed culture-independent studies [51] and metabolomics applied to the GIT bacteria [10,22,52] have helped to increase the knowledge greatly.

The intestinal microbiota differ quantitatively and qualitatively, increasing in number and population diversity along the length of the GIT (Table 2.1). The oral cavity is colonized by a wide array of aerobic, facultative and anaerobic organisms. Whereas the oxidation-reduction potential decreases, more anaerobic bacteria colonize the distal part of the GIT; thus, over 99% of bacteria located in the large intestine are anaerobes [53,54]. After the approximately neutral pH of the oral cavity, the low pH of the stomach (2.5 – 3.5) is destructive to most microbes. Low number of bacteria ($10^3$ CFU/g, Table 2.1) is dominated by Gram-positive bacteria [55]. The proximal part of the small intestine with low pH, bile and pancreatic secretions and a low transit time is still a hostile environment for most microbes. A pH gradient to the distal part of the small intestine allows a higher number ($10^5$ – $10^8$ CFU/g [55]) and more diverse bacteria to colonize. Studies showed that jejunum microbiota is dominated by *Streptococus* and *Proteobacteria*, and the distal ileum by *Bacterioidetes* and *Clostridium [56].*

In humans, the colon is the most predominant site for fermentation (Figure 2.2). Due to the slower transit, rich nutritional environment and high pH the bacterial density and diversity is much higher ($10^9$ – $5x10^{11}$ CFU/g; Table 2.1). The proximal colon is a saccharolytic environment where most bacterial metabolic activity and non-digestible carbohydrate fermentation occur. The pH of the proximal colon is generally lower than the distal part (5-6 and neutral respectively). The reduced pH is considered to be an outcome of carbohydrates fermentation, resulting in the production of Short-Chain Fatty Acids (SCFA) [57]. In the distal colon, availability of carbohydrates decreases and the pH increases. Bacterial metabolism is slower and proteolysis is the dominating process. Despite the vertical difference in the bacterial population, there is also a horizontal stratification, with

different microbial communities inhabitating the intestinal lumen, mucus, crypt spaces and directly adhering to epithelial cells [58].

**Table 2.1 Estimated numbers of major microbial population groups in different segments of the GIT. Table from Holzapfel, 2006 [59].**

| Microbial group | Stomach | Duodenum | Jejunum & Ileum | Colon |
|---|---|---|---|---|
| | $10^1 – 10^3$ CFU/ml | $10^1 – 10^4$ CFU/ml | $10^5 – 10^8$ CFU/g | $10^9 – 5x10^{11}$ CFU/g |
| *Actinomyces* spp. | | | $10^4 – 10^6$ | |
| *Bacteroides-Prevotella-Porphyromonas* group | up to $10^2$ | ca. $10^3$ | $10^4 – 10^7$ | $10^9 – 10^{11}$ |
| *Bifidobacterium* spp. | | | | $10^9 – 10^{10}$ |
| *Clostridium* spp. | | | $10^4 – 10^5$ | $10^8 – 10^9$ |
| *Coprococcus cutactus* | | | | $10^7 – 10^8$ |
| *Enterobacteriaceae* | up to $10^2$ | $10^2 – 10^4$ | $10^3 – 10^6$ | $10^5 – 10^7$ |
| *Enterococcus* spp. | | | $10^2 – 10^4$ | $10^3 – 10^6$ |
| *Eubacterium* spp. | | | | $10^9 – 10^{11}$ |
| *Fusobacterium* spp. | | | $10^3 – 10^5$ | $10^5 – 10^7$ |
| *Lactobacillus* spp. | $10^1 – 10^3$ | $10^2 – 10^4$ | $10^4 – 10^6$ | $10^5 – 10^8$ |
| *Megamonas hypermegas* | | | | $10^7 – 10^8$ |
| *Megasphaaera elsdenii* | | | | $10^7 – 10^8$ |
| *Methanobacteria* | | | | up to $10^4$ |
| *Peptostreptococcus* spp. | | | $10^2 – 10^6$ | $10^8 – 10^9$ |
| *Proteus* spp. | | | | $10^3 – 10^6$ |
| *Pseudomonas* spp. | | | | $> 10^3$ |
| *Staphylococcil* | | | | ca. $10^3$ |
| *Streptococcus* spp. | $10^1 – 10^3$ | | $10^3 – 10^8$ | up to $10^7$ |
| *Veillonella* spp. | | | $10^3 – 10^7$ | $10^5 – 10^8$ |
| Yeasts | | | | ca. $10^3$ |

Most studies exploring the human intestinal microbiota are focused on the fecal microflora due to its easy access and collection. However, it has been postulated that the fecal microbial populations may be distinct and have different properties than the surface-

adherent microbes in the colon mucus [60]. It has to be taken into consideration that the fecal bacterial population is not a representation of the total GIT microflora.

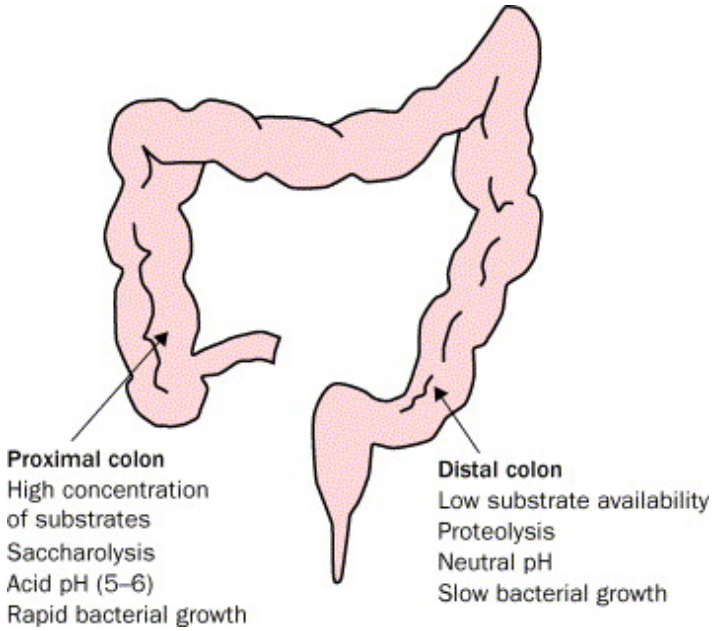


**Figure 2.2 Fermentation in the colon. Figure from Guarner and Malagelada, 2003 [61].**

## 2.3. Metabolic relationship between the host and microflora

Human global metabolism at the whole-body level is the integration between the activities of our genome and the microbiome. As the human GIT provides nutrients to cells and tissue by the circulatory system, so do the metabolic products from microbial flora. In other words, every human cell is somehow influenced by metabolites originating from the gut microbiota [62]. This cross-talk between the microbes and the host includes signaling via low molecular weight metabolites, peptides and proteins.

The most studied metabolic contribution of the intestinal microflora to the GIT of the host is the production of the SCFAs. Complex non-digestible carbohydrates from plant sources are fermented by the gut bacteria, producing mainly acetate, propionate and butyrate. Host recovery of the SCFAs occurs by passive diffusion and mono-carboxylic acid transporters [63]. Increases in SCFA production have been associated with decreased pH, which may reduce potential pathogenic clostridia, decrease solubility of bile acids, increase absorption of minerals, and reduce ammonia absorption by the protonic dissociation of ammonia and

other amines [64-66]. Butyrate is the preferred source of energy for colonic epithelial cells. Absorbed acetate and propionate are delivered to hepatocytes, consuming propionate for gluconeogenesis, and acetate might be used for lipogenesis in colonocytes, hepatocytes and adipocytes [67]. SCFA also act as signaling molecules. Propionate, acetate and in a small extent butyrate and pentanoate could stimulate expression of leptin, a hormone with a pleiotropic effects on appetite and energy metabolism [68]. Acetate is the principal SCFA in the colon, increasing cholesterol synthesis. However, propionate, a gluconeogenerator, has been shown to inhibit cholesterol synthesis. Therefore, substrates that can decrease the acetate**:**propionate ratio, may reduce serum lipids and possibly cardiovascular disease risk [69]. Butyrate has been studied for its role in nourishing the colonic mucosa and in the prevention of cancer of the colon, by promoting cell differentiation, cell-cycle arrest and apoptosis of transformed colonocytes; inhibiting the enzyme histone deacetylase and decreasing the transformation of primary to secondary bile acids as a result of colonic acidification [70]. Therefore, a greater increase in SCFA production and potentially a greater delivery of SCFA, specifically butyrate, to the distal colon may result in a protective effect [69,71]. The production of SCFAs is dependent on the number and types microflora in the colon, type of substrate and the gut transit time [66,72]. Mostly related bacterial taxa, associated with the SCFAs production are Clostridial clusters IV and XIVa, *Eubacterium, Roseburia, Faecalibacterium* and *Coprococcus* [65,67,69].

Colonic bacteria also contribute to the salvage of bile salts, which escape active transport in the distal ileum. The two primary bile acids synthesized in the human liver are cholic acid and chenodeoxycholic acid, conjugated to the bile salts. Ileal bile salt transport is highly efficient (95%), but a small fraction escapes the enterohepatic circulation and becomes substrate for significant microbial biotransformation in the large bowel [73]. The major bile salt modifications in the human large intestine include deconjugation, oxidation of hydroxyl group and dehydroxylation [74]. Deconjugation and dehydroxylation of bile salts increases their hydrophobicity and their $Pk_a$, permitting their recovery by passive absorption across the colonic epithelium. However, the increased hydrophobicity of the transformed bile salts is also associated with increased toxic and metabolic effects. High concentrations of secondary bile acids in feces, blood, and bile have been linked to the pathogenesis of cholesterol gallstone disease and colon cancer [75]. The main secondary

bile acids formed by the interaction of both human and intestinal microbial metabolism are deoxycholic acid and lithocholic acid. Figure 2.3 shows bacterial bile salt-biotransforming reactions present in the human GIT. Studies done with the HBM (human baby flora) colonized mice showed that significant variations in microbial populations lead to modification of bile acid symbiotic metabolism [76]. The deconjugation of bile acids involves the activity of bile salt hydrolase, which is synthesized in significant amounts by

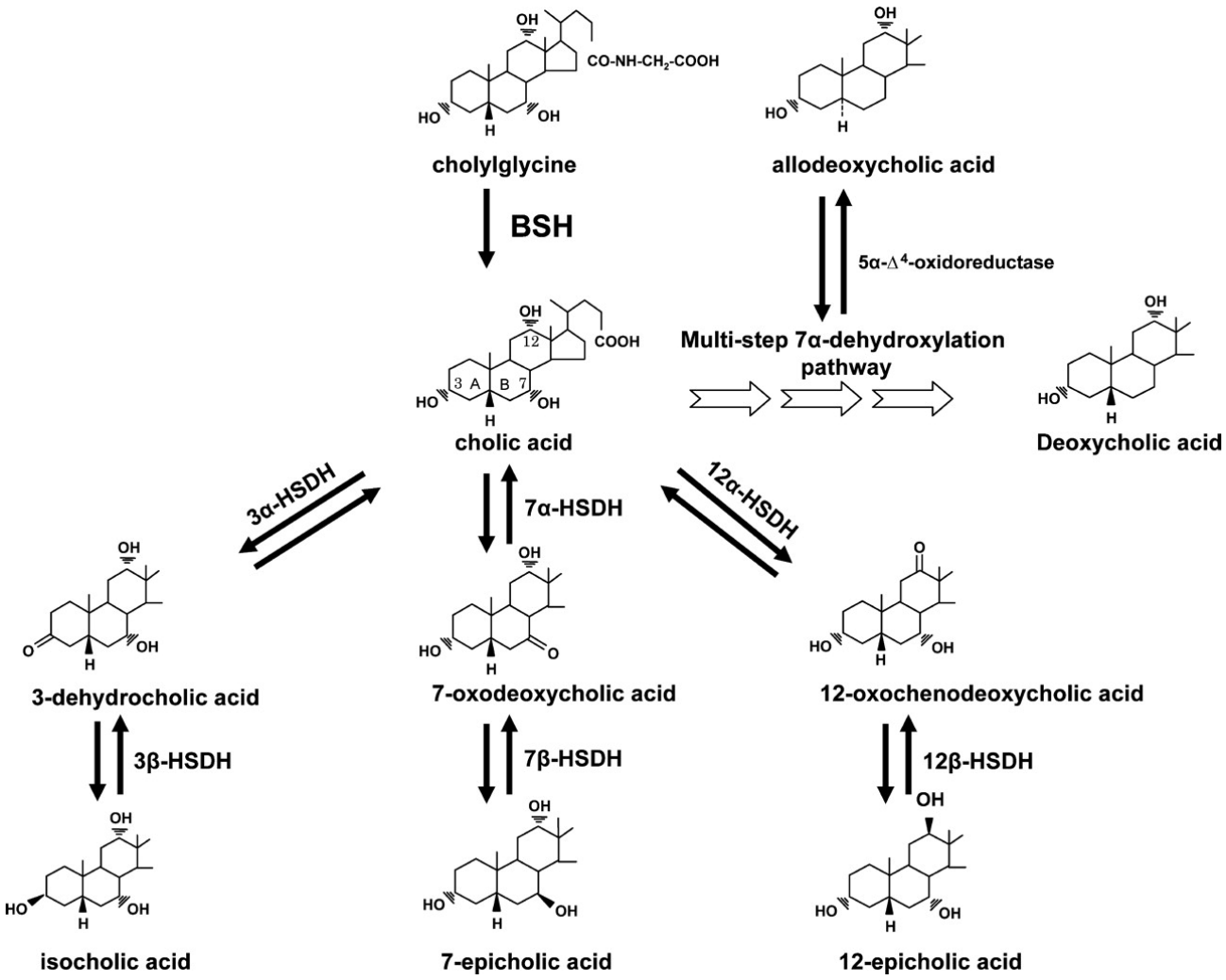


**Figure 2.3 Bacterial bile salt-biotransforming reactions in the human intestinal tract. Hydroxy group carbons of cholate are numbered and the AB rings are identified. The 3, 7, and 12 carbons of cholic acid are numbered. BSH, bile salt hydrolase; HSDH, hydroxysteroid dehydrogenase. Figure from Ridlon, 2006 [74].**

*Lactobacillus* and *Bifidobacterium* [77]. Another study showed that biotransformation of the bile salts was highly influenced by the balance between *Lactobacillus* and *Bifidobacterium and Enterobacteria* and *Bacteroides [78].* Other bacterial genus related to the bile acid co-metabolism are *Clostridium* and *Escherichia* [73,74,79,80].

Bile acids are amphiphilic compounds, and their biotransformation modifies their hydrophobic/hydrophilic balance, which directly relates to lipid emulsification and eventually absorption [81-83]. Recent findings show the essential microbial role in affecting the bile acid-controlled signaling pathways, involved in energy and lipid metabolism [84]. For instance, cholesterol-lowering effects and protection against very-low-density lipoproteins (VLDLs) and low-density lipoproteins (LDLs) oxidation were reported for *Lactobacillus and Bifidobacteria* [85,86]. Other studies indicate microbiota in the GIT can modulate the host's lipid storage and metabolism [87-89]. The symbiotic metabolism between the mammalian host and the bacterial microflora related to the bile acids and lipid metabolism is presented in Figure 2.4.

Liver metabolism is influenced by the microbial biotransformation of choline, which is an essential dietary nutrient. However, intestinal microbiota also convert dietary choline to trimethylamine, which is then further metabolized in the liver to trimethylamine-N-oxide [90]. The final metabolite is known for its negative impact on the cardiovascular system, involvement in the atherosclerosis and nonalcoholic fatty liver disease [22]. Bacteria related to the transformation of choline are *Faecalibacterium prausnitzii* and *Bifidobacterium* [71,91].

Flavonoids are polyphenolic compounds that occur ubiquitously in foods of plant orgin. Variations in the heterocyclic ring divide them into flavonols, flavones, catechins, flavanones, anthocyanidins and isoflavonoids. A multitude of *in vitro* studies have shown that flavonoids can inhibit or induce a large variety of mammalian enzyme systems, involved in important pathways, regulating cell division and proliferation, platelet aggregation, detoxification, and inflammatory and immune response [92]. It has been hypothesized that the antioxidant properties of flavonoids may protect tissues against oxygen free radicals and lipid peroxidation, which might be involved in several pathological conditions [93,94]. Absorption of flavonoids from the diet was long considered to be negligible, as they are present in foods bound to sugars as β-glycosides, except catechins.


**Figure 2.4 Microbe-mammalian metabolic interactions related to bile acid and lipid metabolism. The bacterial reprocessing of the bile acid pool and regulation of bile acids metabolism by bacterial SCFAs significantly affect the enterohepatic recirculation and the systemic lipid metabolism, which are emulsification, absorption and transport of dietary fats. The gut-bacterial-induced regulation of enterohepatic recirculation also leads to a physiological regulation of oxidative stress (glutathione), reprocessing of fatty acids (deposition, apoprotein and VLDL synthesis) and VLDL secretion from the liver, which results in controlling of the influx and efflux of fatty acids in the liver. BA, bile acids; CA, cholic acid; GPC, glycerophosphorylcholine; GSH, glutathione; HBF, human baby flora; LDL, low-density lipoproteins; βMCA, β-muricholic acid; SCFAs, short-chain fatty acids; TβMCA, tauro-β-muricholic acid; TCA, taurocholic acid; VLDL, very low-density lipoproteins. Figure from Martin *et. al*, 2007 [76].**

Only free flavonoids without a sugar molecule (aglycones) were considered to be able to pass the gut wall, as there are no mammalian enzymes, capable of splitting the β-glycosidic bonds [95]. It has been shown, that the final biological activity of flavonoids depends on the intestinal bacterial metabolism, capable not only of breaking the β-glycosidic bonds, but also capable of biotransforming some of the flavonoic compounds, changing their bioactivity [96,97]. Equol was described as an important bacterial metabolite of daidzein [98]. Additionally equol was shown to have increased beneficial health effects compared to daidzein [99]. However, there is also a possibility for microbiome metabolism to convert daidzein into less active O-desmethylangolensin [100]. Inactive prenylated flavonoid, isoxanthohumol can be activated by intestinal microbiota into its active form, 8-pernylnaringenin [101]. Lignans are also polyphenolic compounds present in the human diet in high amounts [102]. Secoisolariciresinol, matairesinol, lariciresinol and pinoresinol are considered as being the most relevant dietary lignans [103-105]. Similarly to the flavonoic compounds, inactive plant lignans can be converted into mammalian lignans (enterolignans); enterodiol and enterolactone by intestinal microbiota [106]. Enterolignanas have estrogen-like biological properties, additionally interacting with various enzymes and proteins. Mentioned activities may result in e.g. protection against breast and colon cancer, and coronary heart diseases [107]. Microbial activation of flavonoids and lignans is shown to be dependent on the individual intestinal microbial community and activity [108]. Given the important role of hydrogen in the intestinal production of equol and enterolignans, the methane-producing and sulfate-reducing bacteria seems to have a central position in this microbial biotransformation [97,109]. Equol production is also related to *Clostridium coccoides-Eubacterium rectal* cluster [108,110,111].

Proteolysis and amino acids fermentation is related to the increase of phenolic compounds in the colon [112], which are usually absorbed and detoxified by glucuronide and sulfate conjugation in the mucosa of the bowel and in the liver. Afterwards, predominantly 4-cresol, phenol and 4-ethylphenol [113] are excreted with the urine [114]. The production of phenolic compounds in mammals is associated with *Clostridium*, *Bifidobacterium, Bacteroides fragilis* and *Escherichia coli* and in many cases high concentrations in the urine are related to a variety of disease states in humans [115]. However, altered amount of these products were observed together with a change in the diversity of microbiota,

such as loss of *Lactobacillus* and *Bacteroidetes* species in case of inflammatory bowel disease and differences in the ratio of the *Firmicutes* and *Bacteroidetes* species in case of weight loss [116,117]. On the other hand, indole derivatives, also aromatic compounds, were associated with a positive impact on the GIT, such as protection against stress-induced lesions, modulation of pro-inflammatory gene expression, increasing expression of anti-inflammatory genes and strengthening of epithelial cell barrier properties [71,118,119]. Additionally batch culture incubations with human fecal bacteria revealed the effect of pH, carbohydrate, protein, peptide and free amino acids availability on the production of phenolic and indolic compounds [115]. Results from this study showed that the type of substrate was an important factor limiting production of phenolic and indolic compounds. With protein (casein), the main end products of amino acid metabolism were phenol, phenylacetate, and phenylpropionate. Peptide fermentation of tryptic and peptic digests resulted in an increase in molar ratios of tyrosine dissimilation intermediates, with a reduction in phenylalanine fermentation. Indole was detected only when its free amino acid precursor was added to the system [115]. In the mammalian GIT amino acids are produced by the digestion of proteins and are mostly absorbed in the upper part of the intestines. However, *in vitro* studies show that some of the amino acids reach the lower part of intestines and may be used by bacterial flora as the source of nitrogen, leading to a production of SCFAs and gases in the colon. On the other hand, proline, threonine, asparagine and arginine were the only amino acids utilized by intestinal bacteria in the mentioned *in vitro* studies [120].

Most primary amines are biosynthesized by decarboxylation from amino acids in the host cells. In the mammalian colon β-alanine, cadaverine, putrescine, tyramine and γ-aminobutyric acid (GABA) are also known to be synthesized by colonic bacterial decarboxylase from free amino acids, aspartic acid, lysine, ornithine, tyrosine and glutamine, respectively [120]. Polyamines, such as putrescine, spermidine and spermine, are one of the most important metabolites produced by intestinal microbiota (*Clostridium*, *Campylobacter* and possibly *Escherichia coli [71,120]), that* affect the health and diseases of the host [121]. As organic cations, they are required for cell growth and differentiation, synthesis of DNA, RNA and proteins and they are absorbed as energy sources from the intestinal lumen [122]. Polyamines serve many functions, such as maturation and

maintenance of intestinal mucosal barrier, anti-inflammatory actions, anti-mutagenicity and autophagy [123-126].

Vitamins are essential for normal cellular functions, growth and development and their deficiency leads to a variety of clinical abnormalities, which range from anemia to growth retardation and neurological disorders. Humans are not able to synthesize most of the vitamins and must obtain these micronutrients from exogenous sources. It has been recognized that gut bacteria, especially *Bifidobacterium*, are able to synthesize some of required vitamins, like vitamin K, B12, biotin, folate, thiamine, riboflavin and pyridoxine [71,127,128].

Many other metabolites related to the intestinal microbiota (e.g. *Bacteroides, Pseudobutyrivibrio, Ruminococcus, Lactobacillus*), such as D-lactate, formate, methanol, ethanol, succinate, lysine, urea, etc were found to influence the mammalian host by direct or indirect synthesis or utilization of compounds or modulation of linked pathways [71,129,130]. Although there is a global understanding of metabolite flow across the microbiome-host-food web, for many reasons, including the difficulty in culturing many of the bacteria form the GIT, our knowledge of bacterial species synthesize which metabolites *in vitro* and co-metabolism between the intestinal species between each other is currently limited. However metabolic profiling as previously described offers an alternative strategy for characterizing GIT human and bacterial metabolites.

# 3. Metabolomics in relation to pro-, pre- and syn-biotics

## 3.1. Introduction to the pro-, pre- and sym-biotic concept

Understanding of the gut microbiology in human health and nutrition has lead to rapid development of the number of pro-, pre- and sym-biotics. Recently, these supplements are aimed not only at the enhancement of well-being, but also as alternative or complementary treatments in acute and chronic diseases.

### 3.1.1. Probiotics

Probiotics are defined as “live microorganisms, which, when administered in adequate amounts, confer a health benefit for the host” [131]. The addition of probiotics to the intestine may optimize the balance of the intestinal microbiota by changing the intestinal pH and producing antimicrobial substances, such as bacteroicins, organic acids, and hydrogen peroxide [132-134]. The increase in beneficial bacteria results in competition with pathogenic bacteria for nutrients, potentially preventing acute diarrheas and pathogenic infections [135-137]. Animal and human studies have shown that probiotics may reduce intestinal permeability, provide nutrition and stimulate proliferation of the colonic cells and participate in the regulation of intestinal functions [138-140], protecting the host from colon cancer [141,142]. Additionally, probiotic bacteria have been shown to increase the host immunological barrier, preventing common infections (e.g. colds and fever), allergic disorders and inflammatory bowel diseases [143-147].

Bifidobacteria and lactobacilli are considered beneficial in the GIT microbiota and their prevalence is generally a good indicator of healthy, balanced microflora. Therefore, most of the microorganisms corresponding to the definition of probiotics are from bifidobacteria and lactobacilli genera. However, other microorganisms have also been tested for their probiotic properties. Probiotic microorganisms alone or in combination with each other are currently available as capsules or powders, or used in the production of various fermented products. Commonly used probiotic bacteria with documented clinical effects are given in Table 3.1.

**Table 3.1 Probiotic bacteria with documented clinical effects. Table adapted from Santosa *et. al*, 2006 and Rouzaud, 2007 [132,148].**

| Bacteria | Reference |
|---|---|
| **Bifidobacteria** | |
| *Bifidobacterium bifidum* | [149] |
| *B. breve* Yakult strain | [150] |
| *B. lactis* Bb-12 | [151] |
| *B. longum* 913 | [9] |
| **Lactobacilli** | |
| *Lactobacillus acidophilus* NCFM | [152] |
| *L. acidophilus* LA-1 | [153] |
| *L. acidophilus* LB | [154] |
| *L. casei immunitass* DN114001 | [155] |
| *L. casei Shirota* YIT 0918 | [156] |
| *L. gasseri* | [157] |
| *L. johnsonii* La1 | [158] |
| *L. plantarum* 299v | [159] |
| *L. reuteri* | [160] |
| *L. rhamnosus* GG (ATCC 53103) | [161] |
| *L. bulgaricus* | [162] |
| **Other bacteria** | |
| *Enterococcus faecium* | [163] |
| *Escherichia coli* Nissle 1917 | [164] |
| *Streptococcus salivarius* subsp. *thermnophilus* | [165] |
| **Yeasts** | |
| *Sacchromyces boulardii* | [166] |

### 3.1.2. Prebiotics

As previously mentioned, non-digestible dietary carbohydrates, which escape digestion in the upper part of the GIT, become available as growth substrates for the colonic microbiota. In the human diet the majority of these carbohydrates are plant cell wall polysaccharides, such as cellulose, arabinoxylan, xyloglucan, β-glucan, mannan, pectins and lignin [167,168]. However, it has been shown that only a certain types of bacteria have the enzymatic capability to utilize specific plant polysaccharides [169-173]. Therefore, beside the probiotic benefits, non-digestible dietary carbohydrates are another approach to confer the health benefits of intestinal beneficial bacteria by increasing the numbers of bifidobacteria and lactobacilli at the expense of other bacterial groups, additionally stimulating the saccharolytic activity in the colon [46].

The concept of prebiotics is defined as ”selectively fermented ingredients that allow specific changes, both in the composition and/or activity of the gastrointestinal microbiota that confers benefits upon host well-being and health” [174]. According to this definition, inulin, fructo-oligosaccharides (FOS), galacto-oligosaccharides (GOS), trans-galacto-oligosaccharides, gluco-oligosaccharides, soybean oligosaccharides, isomalto-oligosaccharides, lactosucrose and lactulose have been classified as prebiotic substances [148]. Many studies focus on finding the new candidates to fulfill the requirements, namely focusing on gentio-oligosaccharides, chito-oligosaccharides, xylo-oligosaccharides (XOS), arabino-xylo-oligosaccharides, arabino-oligosaccharides, oligodextrans, pectic-oligosaccharides, arabino-galacto-oligosaccharides, rhamno-galacturo-oligosaccharides, galacturonic-oligosaccharides and sialic acid oligosaccharides [148,174,175]. Selective stimulation of bifidobacteria and lactobacillus by non-digestible carbohydrates is affected by their chemical structure - type of glycosidic linkage, degree of branching and degree of polymerization (DP). Additionally, size of the carbohydrate influence where in the colon the fermentation occurs. Carbohydrates with low DP reach the proximal colon (Figure 2.2), where number of bacteria and substrate concentration is high. Non-digestible carbohydrates with high DP might reach the distal colon [176,177].

The possible beneficial effects of prebiotics include the control of intestinal transit time and bowel habits, reduction of the risks of atherosclerosis, osteoporosis, obesity, type-2 diabetes, cancer, infections and allergies. However, most studies providing information

about the beneficial effect of prebiotics are based on animal models and the effect in humans is still controversial [178].

### 3.1.3. Synbiotics

The concept of synbiotics is a combination of probiotic and prebiotic approaches to confer benefits upon host well-being and health. A synbiotic aims at stimulating the growth and/or activity of beneficial intestinal microbes by using an appropriate non-digestible carbohydrate in conjunction with one or several probiotic strains [148]. Synergistic action of pre- and probiotics has been observed in animal studies with inulin and *Bifidobacterium longum*, trans-oligosaccharides and *Bifidobacterium breve*, FOS and *Bifidobacterium* [179-181]. Clinical trials were performed with probiotic bifidobacteria and lactobacillus in combination with GOS, FOS and inulin, resulting in beneficial health effects on the participants, similar to the effects related to both dietary supplements [148]. Additionally, it has been postulated that prebiotics may provide protection of probiotics during intestinal transit and/or enhance their growth as well as that of the targeted commensal populations [182,183].

## 3.2. Metabolic alterations induced by pro-, pre- and syn-biotics in the host

By changing the composition and functionality of the microbiota, co-metabolism between different bacteria and the host may also be affected. The impact of a functional food supplements on the function of the intestinal population is not fully understood. However, an increasing number of studies dedicated to the metabolic alterations induced by pro-, pre- and syn-biotics is slowly putting the pieces of the puzzle together.

The metabolic effects of *Lactobacillus rhamnosus* and galactosyl-oligosaccharides given alone or as a synbiotic to mice colonized with human baby microbiota were studied by Martin *et. al* [184]. Acetate production was increased due to the stimulation of *Bifidobacteria* growth in presence of the prebiotic [7]. Altered transmethylation metabolic pathways were observed, interconnecting phosphocholine, betaine, dimethylgycine, sarcosine, choline, betaine and the formation of methionine from homocysteine, in liver

and pancreas. These results suggest that functional prebiotics might help with the metabolism homeostasis and detoxification processes [185,186]. This hypothesis is supported by the presence of carnitine and acetyl-carnitine in higher amounts in the urinary excertions [187,188]. Prebiotic supplementation has been shown to significantly reduce triglycerides in the liver, which could have an effect on the whole system lipid metabolism [189-192]. In the same studies, *Lactobacillus rhamnosus* supplementation resulted in changes of bacterial microflora. Metabolomic analysis showed the probiotic potential of lipoprotein reduction in the plasma as well as lower level of glutamine and glycogen [11]. Indirectly, the level of ascorbate in the body, a crucial cofactor for catecholamine biosynthesis, antioxidation and adrenal steroidogenesis might also be affected by the probiotic administration [193,194]. Supplementation of both *Lactobacillus rhamnosus* and galactosyl-oligosaccharides resulted in complementary effects of pro- and pre-biotics, at both bacterial and host metabolic levels. *Bifidobacterial* growth was increased more significantly than in case of the two supplements separately. From the methabolic point of view, as SCFAs were not affected compared to the prebiotics alone and a more significant reduction of liver triglycerides, kidney and plasma lipids was observed in case of synbiotic administration. Other studies done by Martin *et. al* [11,12] with a combination of *Lactobacillus paracasei*, *L. ramnosus* and two galactosyl-oligosaccharide in the HBM mice, gave the similar results. The results showed changes in lipid profiles, gluconeogenesis, amino-acid and the methylamine metabolism associated with fermentation of carbohydrates by different bacterial strains.

Studies presented by Martin *et. al* are performed in the animal model and the given results could differ in case of the human supplementation. On the other hand, studies focused on the synbiotics showed an increase in HDL and a decrease in LDL/HDL cholesterol in the clinical trials via administration of yogurt containing FOS, *Bifidobacterium longum* and *Lactobacillus acidophilus* [9]. Administration of similar synbiotic products to healthy individuals resulted in significant alterations of the metabolic activity of the intestinal microbiota [13]. Among more than 150 molecules occurring in the profile of fecal matter, amino acids and SCFAs were the most affected by the treatment. General increases in the SCFA content has been observed in parallel with a significant decrease of amino acids content, both in the aromatic (phenylalanine and tyrosine) and aliphatic regions. The significant decrease of amino acids, which was not substituted by the presence of their

toxic metabolites, such as ammonia and amines, suggests that the synbiotic food favored the amino acid assimilation rather than their catabolization. In fact the amino acids would not be required for energy due to the availability of FOS. Greater carbohydrate availability avoided the accumulation of toxic by-products of amino acid fermentation [195,196]. The increase of the SCFAs arises from the metabolism of FOS and amino acids.

Significant increase in the common SCFAs production by the administration of GOS with *Bifidobacterium breve* and *Lactobacillus casei* was found in the infant [197]. Synbiotic food supplement in another human trial, containing FOS, *Lactobacillus helveticus* Bar13 and *Bifidobacterium longum* Bar33, was shown to not only increase the production of SCFAs, but also ketones, carbone disulfate and methyl acetate in the fecal matter, that regulate cell proliferation, differentiation, anti-inflammatory and chemopreventative properties as well as detoxification processes [198].

The presented studies show the breadth and the depth of gut microbiome modulation of host biochemistry and reveal that major mammalian metabolic processes are under symbiotic homeostatic control, with a probability to modulate via intake of the functional food supplements.

# Paper published

## Metabolic footprint of Lactobacillus acidophilus NCFM at different pH

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ORIGINAL ARTICLE

# Metabolic footprint of *Lactobacillus acidophilus* NCFM at different pH

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**Abstract** *Lactobacillus acidophilus* NCFM is a well known microorganism from the genomic and probiotic point of view. In order to analyze the potential interactions of NCFM with the surrounding environment, in vitro tests with the metabolic footprinting approach were performed. It was found that NCFM increased the concentration of lactic acid, succinic acid, adenine and arginine in the medium. The metabolism of NCFM did not change significantly between pH 5 and 7, suggesting that other environmental factors than pH might have bigger impact on its colonization throughout the gastrointestinal tract.

**Keywords** *Lactobacillus acidophilus* NCFM · Metabolomics · Metabolite footprint · GIT · Probiotics · LCMS

## 1 Introduction

The human organism lives in a symbiotic relationship with a very complex bacterial ecosystem. Around $10^{14}$ bacterial cells inhabit the gastrointestinal tract (GIT) (Lebeer et al. 2008), and are prone to communicate with each other and with the host in order to keep the balance of the vast ecosystem that they constitute (O'Flaherty and Klaenhammer 2010). This type of bacterial community exhibits several levels of interaction, e.g. through protein production, enzymatic activity, DNA and RNA exchange as well through excretion of small molecules, metabolites (Ben et al. 2004), on which this paper will focus.

During the last decade, it has become evident that microbiota in the human gut plays an important role for human health (Mazmanian et al. 2005; Turnbaugh et al. 2006; Rafter 2003). A large number of studies have addressed the effects of dietary interventions on the presence of specific bacterial metabolites that are anticipated to play a role for gut health (Lebeer et al. 2008). Such metabolites are produced as a result of life sustaining metabolism, bacterial response to environmental conditions, as well as of microbe-microbe and microbe-host communication (Holzapfel 2006).

The aim of this study was to analyze the potential role of extracellular metabolite production by microorganisms in the gut. The homofermentative *Lactobacillus acidophilus* NCFM was been chosen as an example of a well-described probiotic microorganism, which has been used in many food products and supplements since 1972 (Sanders and Klaenhammer 2001). In silico studies of the complete genome sequence of the NCFM strain revealed significant similarities to other probiotic lactic acid bacteria (Altermann et al. 2005). Similar to other closely related species from the acidophilus group (*L. johnsonii* and *L. gasseri*), NCFM lacked biosynthetic capacity for most vitamins, amino acids and cofactors, but encoded considerable transporter and fermentative capacities, which were expected to be present in organisms inhabiting the nutrient rich conditions of the upper GIT (Altermann et al. 2005).

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While many studies have addressed gene expression in Lactobacillus (Bron et al. 2004a, b; de Vos et al. 2004) and its human host (Hanniffy et al. 2004) during colonization, only a few of them focused on bacterial communication with the surrounding environment through metabolite production (Martin et al. 2007; Wikoff et al. 2009).

Metabolic footprinting is one of the approaches used in metabolomics (Villas-Boas et al. 2007; Mapelli et al. 2008) and focuses on the analysis of extracellular metabolites by use of mass spectrometry combined with multivariate data analysis. Metabolic footprinting provides knowledge about only a small part of the entire bacterial metabolome; however it provides key information that can contribute to the understanding of bacterial communication with the surrounding environment. In order to analyze the potential of the probiotic NCFM to interact with the GIT environment through metabolite production, in vitro tests were performed at three different pH values mimicking the pH that will be encountered by bacteria in the intestine (Warberg 2001). Extracellular metabolites were measured by metabolic footprinting, and the applicability of this method for our purpose was evaluated. Data analysis focused on metabolites, which could be responsible for probiotic properties of the studied strain.

## 2 Materials and methods

### 2.1 Strain preparation

NCFM was kindly provided by Danisco A/S as a frozen culture. Bacteria were taken from cryotubes and grown on deMan-Rogosa-Sharpe (MRS, Oxoid Ltd., Basingstoke, Hempshire, England) agar under anaerobic conditions at 37°C, overnight. Selected colonies were grown in 10 ml of MRS broth (MRS, Oxoid Ltd., Basingstoke, Hempshire, England) under the same conditions, overnight prior fermentations as described below.

### 2.2 Minimal medium

Medium for the fermentation tests was based on the semi-synthetic medium by Barrangou et al. (2003) and consisted of: 1% buffered peptone water (wt/vol.) (Oxoid Ltd., Basingstoke, Hempshire, England), 0.5% yeast extract (wt/vol.) (Oxoid Ltd., Basingstoke, Hempshire, England), 0.2% dipotassium phosphate (wt/vol.) (Merck KGaA, Darmstadt, Germany), 0.5% sodium acetate (wt/vol.) (Merck KGaA, Darmstadt, Germany), 0.2% ammonium citrate (wt/vol.) (Sigma Chemical co., St. Louis, Missouri, USA), 0.02% magnesium sulfate (wt/vol.) (Merck KGaA, Darmstadt, Germany), 0.005% manganese sulfate monohydrate (wt/vol.)(Merck KGaA, Darmstadt, Germany), 0.1% Tween 80 (vol./vol.) (VWR International, Fontenay-sous-Bois, France), and 1% glucose (wt/vol.) (Merck, KGaA, Darmstadt, Germany). pH of the medium was adjusted to: 3, 5 and 7 respectively with sterile solutions of HCl and NaOH. Without carbohydrate supplementation, the medium (at pH 7) did not sustain bacterial growth above $OD_{600} \approx 0.3$ (data not shown).

### 2.3 Fermentation tests

Fermentation tests were done using a *starting* pH of 3, 5 and 7, all in triplets, at 37°C in an anaerobic chamber (Don Whitley Scientific Limited, MACS500, Shipley, West Yorkshire, England). The gas mixture in the chamber contained 10% $H_2$, 10% $CO_2$ and 80% $N_2$. Test tubes with growth medium were placed in the anaerobic chamber overnight, prior to the fermentation, to remove the oxygen. Medium was inoculated with 1% (vol./vol.) of an overnight culture of NCFM grown in MRS broth as described. CFU (MRS agar, 48 h, anaerobic, 37°C) and respective $OD_{600}$ values were obtained. The appropriate medium, kept under anaerobic conditions was used as a blank for $OD_{600}$ measurement. At the end of each test, control plating on Luria–Bertani (LB; Oxoid Ltd., Basingstoke, Hempshire, England; aerobic, 24 h, 37°C) agar was performed in order to exclude the possibility of contamination during the test. After 24 h of fermentation, pH values of the fermentation tests (designated F3, F5 and F7, respectively) and medium without inoculum (designated M3, M5 and M7, respectively), were measured.

### 2.4 Metabolites quenching

After 24 h of fermentation, the cells were spun down at $3{,}000 \times g$ for 15 min at 4°C. 800 µl of supernatants were quickly separated from cells and transferred into 800 µl of cold methanol (stored at −80°C) (Fluka, Sigma-Aldrich, Steinheim, Germany) in triplets in order to quench the metabolism. Samples were stored at −80°C until further analysis. MQ water was stored in the same way as the samples to detect possible contamination.

### 2.5 Metabolite detection

Samples from fermentation tests were analyzed by both Direct Infusion Mass Spectrometry (DIMS) and Liquid Chromatography coupled to Mass Spectrometry (LCMS).

The settings for DIMS in positive and negative mode were: flow 50 µl/min, solvent 50% methanol (Fluka, Sigma-Aldrich, Steinheim, Germany) with 0.1% (vol./vol.) formic acid (Fluka, Sigma-Aldrich, Steinheim, Germany), scan range 50–800 *m/z*, capillary voltage 4,000 V, nebulizer pressure 1.5 bar, dry gas flow (nitrogen) 5.0 l/min, dry

gas temperature 200°C. Analysis was performed on a Brucker microTOFq time of flight mass spectrometer equipped with an electrospray interphase (Brucker Daltonics, Bremen, Germany). Data was acquired for 2 min for each sample. External calibration was done using sodium formate clusters (Sigma-Aldrich, Steinheim, Germany).

Samples from the fermentation tests were analyzed by LCMS as follow: an Agilent Technologies model 1200 liquid chromatigraph (Agilent Technologies, Wallbronn, Germany) coupled to the previously mentioned Brucker microTOFq mass spectrometer. Analytes were separated on Kinetex pentafluorophenyl column 50 × 2.10 mm, 2.6 µl, 100 Å (Phenomenex, USA), using the solvent system: A, 10 mM ammonium formate pH 3.5, and B, acetonitrile. Solvent programming was isocratic 0% B to 0 min followed by a linear gradient to 100% B at 7 min and 100% B at 8 min. Flow rate was 0.25 ml/min at 0 min, increased to 0.4 ml/min at 7 min. Solvent composition and flow were returned to initial conditions at 8.2 min. The oven temperature was 40°C. Injection volumes were 1 µl. The following electrospray interphase settings were used: nebulizer pressure 2 bar, drying gas 10 l/min, 200°C, capillary voltage 4000 V. Scan range was from 50 to 800 *m/z*. Samples were analyzed in both positive and negative mode.

### 2.6 Multivariate data analysis

The differences in metabolite profiles at different pH values in the fermentation tests were evaluated by principal component analysis (PCA). Data was grouped into buckets according to the mass/charge (*m/z*) ratio and time of detection/elution from the column. Size of the buckets was dependent on the MS method used. Data was mean centered and normalised by the sum of buckets. PCA models were calculated using Profile Analysis, versions 1.0 and 2.0 (Brucker Daltonics, Bremen, Germany).

A PCA model of the DIMS in positive and negative (Fig. 2) mode was set up from 0 to 120 s with each bucket size of 60 s. Starting *m/z* value was 50 up to 800 with each size of the bucket 1. Decreasing the size of buckets had no effect on group separation or loading plots (data not shown).

A PCA model of the LCMS in positive mode was set up from 30 s (0–30 s internal calibration) to 600 s with each bucket size of 30 s. Starting *m/z* value was 50 up to 800 with each size of the bucket 1. In positive mode buckets 45 s:203.5 *m/z* (glucose), 75 s:136.5 *m/z*, 45 s:118.5 *m/z*, 375 s:309.5 *m/z* and 135 s:120.5 *m/z* (noise) were excluded from PCA (Fig. 3a).

A PCA model of the LCMS in negative mode was only done on chromatographic data from 30 s (0–30 s internal calibration) up to 420 s, thereby avoiding problems with high noise at the end of each chromatogram. The size of each bucket was 30 s as in positive mode. Starting *m/z* values were from 50 up to 800 with each size of the bucket 0.5. Analyses of 113.05, 231.05, 249.05 and 155.05 *m/z*, creating high noise throughout all chromatogram, were removed from the analyses (Fig. 3b).

In order to confirm the PCA method with bucketing, done in Profile Analysis 1.0, calculations were repeated in MATLAB (MathWorks) software, where the same result was obtained (not shown). Additionally, data from DIMS and LCMS were autoscaled (van den Berg et al. 2006), which only increased the noise, so this approach to the data was disregarded.

### 2.7 Chromatograms analysis

Chromatogram data has been analysed in order to estimate the most probable number of metabolites, using Molecular Features in the Compass DataAnalysis 2.0 software (Brucker Daltonics, Bremen, Germany). Parameter settings for each method were: DIMS—chromatogram scan range (CSR) 0.3–0.9 min, S/N threshold (S/N) 10; LCMS—CSR 0.5–1.2 min, S/N 15. Correlation coefficient threshold 0.7, minimum compound length 3 and smoothing width 1 were the same for both MS methods. CSR for each method was chosen for a chromatogram range in the Base Peak Chromatogram with the most defined peaks, excluding internal calibrants. S/N ratio for DIMS and LCMS was set accordingly to the noise level observed in each method.

Extracted Ion Chromatograms in the DataAnalysis software were performed for selected compounds found to explain the main differences between samples in the PCA analysis.

### 2.8 Metabolite search and confirmation

To assist the identification of the detected metabolites by DIMS and LCMS, the Human Metabolome Database (HMDB; Wishart et al. 2009) was used together with the Kyoto Encyclopedia of Genes and Genomes Pathway Database (KEGG; Kanehisa and Goto 2000). Molecular formulas of the metabolites from LCMS were generated, basing on exact mass and isotopic composition with Brucker Daltonics Software. The identity of the metabolites was confirmed by commercial standard compounds by: (1) LCMS retention times and exact mass; (2) by co-elution of metabolites and standards in spiked samples. Standard compounds used were: Succinic Acid (Sigma-Aldrich, Steinheim, Germany), DL-Lactic Acid (Fluka, Sigma-Aldrich, Steinheim, Germany), L-Arginine (Sigma, Sigma-Aldrich, Steinheim, Germany), Adenine (Sigma Grade, Sigma, St. Louis, MO, USA) and Adenosine (Sigma Grade, Sigma, St. Louis, MO, USA).

2.9 Statistics

*P*-values were calculated by ANOVA—Single Factor, with the alpha factor of 0.05 for different groups of samples (Table 3). Data used for calculations were normalised by sum of bucket intensities from PCA on LCMS analyses. As the noise had a high impact on the data from LCMS measurements, the $P > 0.001$ was considered to have no significant differences.

2.10 Live/dead cell staining of NCFM

Differentiation between live and dead bacterial cells was done by use of LIVE/DEAD BacLight Bacterial Viability Kit (Molecular Probes, Inc., Eugene, OR), according to the manufacturer's instructions.

## 3 Results and discussion

3.1 Results

*3.1.1 Probiotic NCFM growth in semi-synthetic medium with different pH value*

The growth of NCFM was similar in media with starting pH values of 5 (F5) and 7 (F7) (Fig. 1a). As expected, a starting pH value of 3 (F3) was too low for NCFM to grow and the number of CFU was decreasing during the fermentation (Fig. 1b). During all tests, pH values dropped (Table 1) and the number of live NCFM started to decrease after 6–7 h of fermentation in F7, and after 8–24 h in F5. Most probably bacterial growth was reduced due to the limited amount of required nutrients in the medium, as the pH did not reach very low values (in F7, Table 1) and glucose was still present in the medium after 24 h of fermentation (which was seen in the MS analyses). Comparing the numbers of bacteria (CFU) and $OD_{600}$ values (Fig. 1) showed that at the end of the experiment, a part of the bacterial population was dead. This was also confirmed by Live&Dead staining (data not shown).

Anaerobic conditions also had some effect on the sterile medium itself (M) as the pH level decreased after 24 h of storage in the anaerobic conditions (Table 1). Control tests excluded contamination.

*3.1.2 Metabolite detection and separation*

Initially, the samples were analysed by DIMS as a quick and easy way of acquiring metabolite profile. PCA of the data showed a clear grouping of data from fermentation samples (F3, F5 and F7) as well as of the sterile medium (M3, M5 and M7) according to the pH value (Fig. 2).

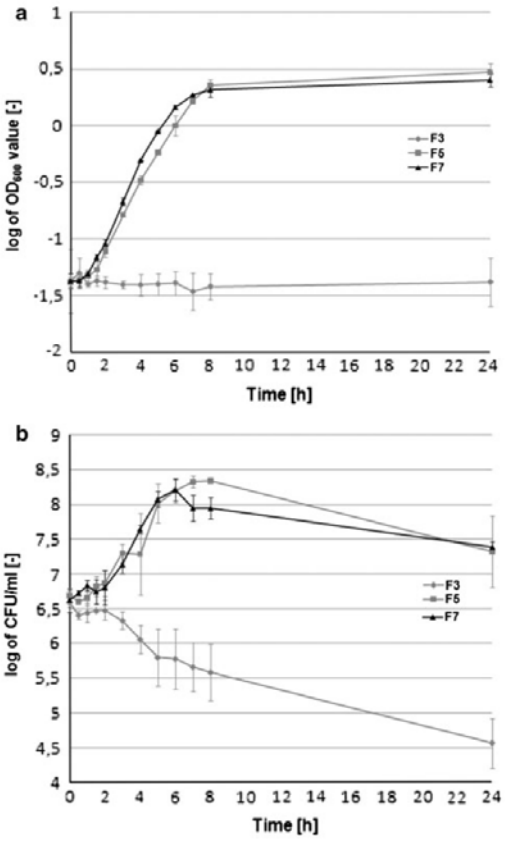


**Fig. 1** Growth of NCFM as $OD_{600}$ value (a) and by CFU/ml (b) in given time. Each point represent an average of three measurements. Error bars designate standard deviations. F3, F5 and F7 designate fermentation tests with different starting pH of 3, 5 and 7, respectively

**Table 1** pH values of fermentation samples (F) and medium kept in anaerobic conditions (M) at the beginning (time 0) and at the end (time 24) of the test

| Sample | Time 0 | Avg. Time 24 | St.Dev. Time 24 (%) |
|---|---|---|---|
| M3 | 3.06 | 2.93 | 3.2 |
| F3 | 3.06 | 2.96 | 3.1 |
| M5 | 5.09 | 5.05 | 1.7 |
| F5 | 5.09 | 3.93 | 13.8 |
| M7 | 6.95 | 6.70 | 0.6 |
| F7 | 6.95 | 4.42 | 6.1 |

However, compounds found to influence the group separation (Fig. 2, right plot) were not further analysed due to the lack of possibility of internal calibration. Estimated numbers of compounds found in the DIMS chromatogram were up to 114 in the positive mode and 87 in the negative

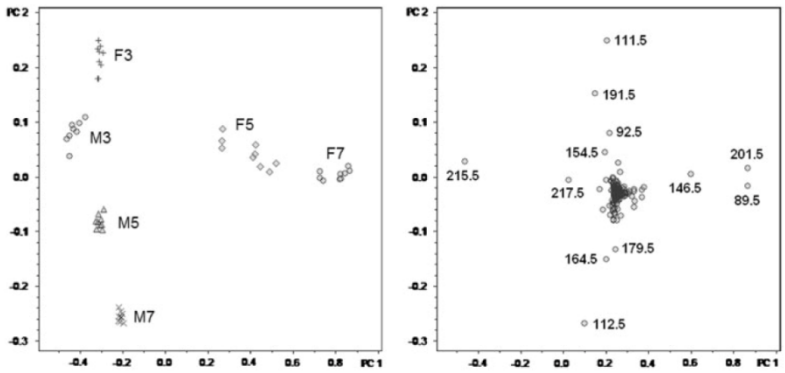


**Fig. 2** Principal Component Analysis of fermentation and medium samples based on DIMS in negative mode. PC1 (81.6%) and PC2 (8.8%) are shown. Score (*left*) and loading (*right*) plots are shown. M3, M5 and M7 designate—media with pH values of 3, 5 and 7 respectively, kept under anaerobic conditions. F3, F5 and F7 designate fermentation samples with starting pH values of 3, 5 and 7, respectively

mode. For detailed analyses, LCMS delivered a better metabolite separation and internal calibrant introduction for higher mass accuracy. Extracted ion chromatograms were created for the ions explaining most of the group separation in the PCA plots. Only ions showing a clear chromatographic peak in data files, where bucket statistics had shown the highest response, were subjected to further analysis. Ions which appeared significant only as a result of a higher noise level in some data files were disregarded, and are not labeled in the loading plots in the Fig. 3. Estimated numbers of compounds found in the LCMS chromatograms were up to 100 in the positive mode and 60 in the negative mode.

Chemical data analyzed by PCA showed the compounds differences between different starting pH value of the fermentation test and medium, which are shown in Table 2. For adenine, arginine and succinic acid these differences were mainly quantitative—those metabolites were present

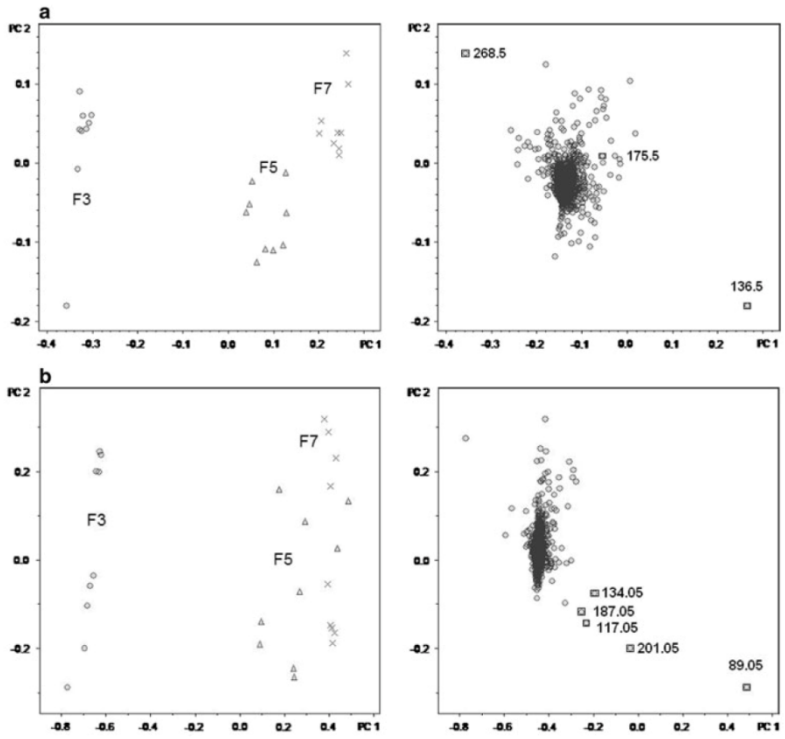


**Fig. 3** Principal Component Analysis of fermentation samples based on LCMS data. Score (*left*) and loading (*right*) plots are shown. F3, F5 and F7 designate fermentation tests with different starting pH of 3, 5 and 7 respectively. Marked variables correspond to the metabolites with described mass (Table 2). Given **a** positive mode, showed PC1 (65.4%) and PC2 (7.5%); and **b** negative mode, showed PC1 (41.6%) to PC2 (6.4%)

in all samples, but in different amounts. Adenosine, lactic acid and an unknown compound were the only metabolites found as qualitative differences between samples—adenosine present only in F3 as well as all M3, M5 and M7; lactic acid only in F5 and F7. Table 3 shows the *P*-values obtained by comparison of samples and a significant difference between all metabolites present in F3 and in either F5 or F7, respectively is seen. Between F5 and F7, the only significant difference is the level of arginine. Apparently, extracellular metabolite production at a level distinguishable from the media background (and matrix effects) does not occur at pH 3, since there was no significant difference between M3 and F3 (Table 3).

There was one single metabolite, which we were not able to identify (Figs. 3b, 4b). This unknown metabolite was eluting at the same time and with approximately the same ratio between sample intensities as lactic acid (Fig. 4), so we speculate that it could be an adduct of this acid. However, as many defined compounds (Fig. 4) were eluting at the beginning of the chromatogram, other types of compounds can not be excluded.

## 4 Discussion

In vitro fermentation followed by metabolomic analysis tools revealed differences in the metabolite production of NCFM, as a function of the surrounding pH value. The medium with pH 3 did not sustain NCFM growth; however after 24 h live bacteria were still present in the suspension, indicating that these bacteria are able to survive the harsh conditions present in stomach and duodenum, as well as other Lactobacillus strains (Pitino et al. 2010). No extracellular metabolites were observed when comparing samples F3 to M3 (PCA for LCMS data—plots not shown), which indicates that no "stress" related compounds, were produced under these acidic conditions. What is also important for a probiotic strain is that the growth and metabolic activity of NCFM at pH 3 did not show signs of acidogenicity (production of acid at low pH) and aciduric ance (capacity to function at low pH) (van de Guchte et al. 2002).

NCFM growth at pH 5 and pH 7 was very similar (Fig. 1) and the same is true for the metabolites seen. This

**Table 2** Identified metabolites, which differ between F3, F5 and F7 (Fig. 3)

| Name | Adduct | $M_{PCA}$ (Da) (Fig. 3) | $M_{LCMS}$ (Da) | $M_{ST}$ (Da) | $M_{HMDB}$ (Da) | Error (mDa) |
|---|---|---|---|---|---|---|
| Adenine | M + H [1+] | 136.5 | 136.0607 | 136.0624 | 136.061768 | 1.1 |
| | M − H [1−] | 134.05 | 134.0467 | 134.0454 | 134.047211 | 0.5 |
| Adenosine | M + H [1+] | 268.5 | 268.1021 | 268.1010 | 268.104004 | 1.9 |
| Arginine | M + H [1+] | 175.5 | 175.1179 | 175.1177 | 175.118960 | 1.1 |
| Lactic acid | M − H [1−] | 89.05 | 89.0227 | 89.0193 | 89.024414 | 1.7 |
| | 2M + Na − 2H [1−] | 201.05 | 201.0327 | – | – | – |
| Succinic acid | M − H [1−] | 117.05 | 117.0182 | 117.0172 | 117.019333 | 0.6 |
| Unknown | – | 187.05 | 187.0425 | – | – | – |

$M_{PCA}$ designates mass taken from PCA; $M_{LCMS}$ designates mass taken from LCMS analysis of test samples; $M_{ST}$ designates mass taken from LCMS analysis of standard compounds; $M_{HMDB}$ designates mass given by Human Metabolome Data Base; 'Error' designates the mass difference between measured $M_{LCMS}$ and found $M_{HMDB}$

**Table 3** *P*-value between sets of samples F (fermentation samples) and M (sterile medium kept under anaerobic conditions) with different starting pH values (3, 5 and 7)

| Name | $M_{PCA}$ (Da) (Figs. 3, 4) | *P* value | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | F3vsF5 | F3vsF7 | F5vsF7 | F3vsM3 | F5vsM5 | F7vsM7 | M3vsM5 | M3vsM7 | M5vsM7 |
| Adenine | 136.5 | 6.56E-17 | 4.7E-16 | **0.0660** | **0.0898** | 3.86E-17 | 8.99E-16 | **0.9366** | **0.5088** | **0.3048** |
| | 134.05 | 1.23E-12 | 1.82E-17 | **0.9301** | **0.0046** | 1.33E-12 | 3.11E-17 | **0.0226** | **0.0032** | **0.4972** |
| Adenosine | 268.5 | 1.34E-16 | 9.6E-18 | **0.0023** | **0.0072** | 4.85E-19 | 1.17E-25 | **0.0096** | 4.82E-05 | **0.0561** |
| Arginine | 175.5 | 1.66E-08 | 8.39E-11 | 5.25E-05 | **0.0313** | 2.15E-10 | 7.36E-12 | **0.2671** | **0.3853** | **0.4529** |
| Lactic acid | 89.05 | 2.32E-11 | 1.51E-18 | **0.2269** | **0.0018** | 2.84E-11 | 2.79E-18 | 1.49E-05 | 3.16E-08 | **0.0016** |
| | 201.05 | 2.69E-11 | 1.97E-13 | **0.0025** | **0.3607** | 4.4E-11 | 2.19E-13 | **0.0570** | **0.1144** | **0.3746** |
| Succinic acid | 117.05 | 1.46E-10 | 4.87E-12 | **0.0017** | **0.0043** | 4.58E-10 | 2.35E-09 | 1.78E-05 | 4.4E-07 | **0.0068** |
| Unknown | 187.05 | 1.38E-12 | 1.69E-08 | **0.1055** | **0.0056** | 2.41E-13 | 7.33E-08 | 2.16E-07 | 1.25E-05 | **0.5295** |

Data used for calculations were normalised by sum of bucket intensities from PCA on LCMS analysis (Fig. 4). Bold values show no significant difference taken as $P > 0.001$

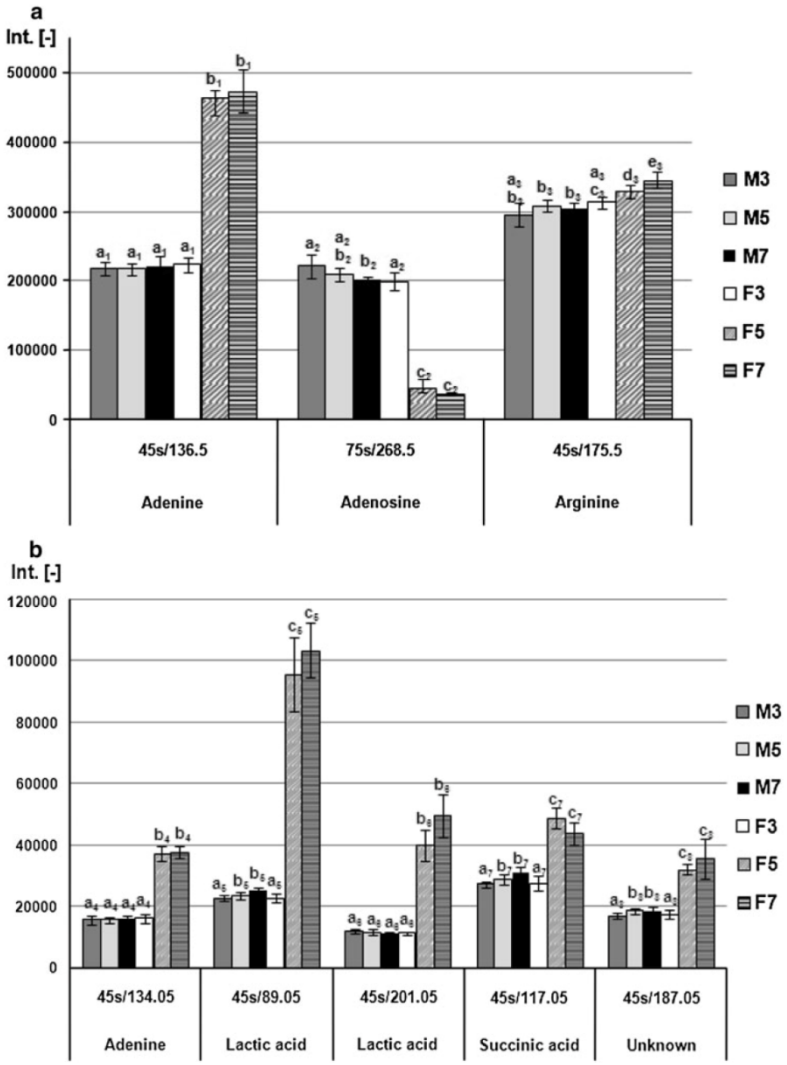


**Fig. 4** Average intensity of given buckets, in which metabolites are present. M3, M5 and M7 designate—media with pH values of 3, 5 and 7 respectively, kept under anaerobic conditions. F3, F5 and F7 designate fermentation samples with starting pH values of 3, 5 and 7, respectively. Due to different scale of intensity in positive (**a**) and negative mode (**b**), data are presented on separate graphs. Data were taken from the PCA, calculated the same way as described in Sect. 2, e.g. by multivariate data analysis, disregarding normalisation by sum of buckets. Each bar represents the average of nine measurements. Error bars designate standard deviations. Different labels ($a_x$, $b_x$, $c_x$, $d_x$ and $e_x$) indicate significant difference (Table 3) between samples for each metabolite separately

suggests that bacterial growth and metabolite production are not affected by fluctuations in pH values between 5 and 7. The differences in abundancy of *Lactobacilli* in sections of the GIT with different pH levels (Holzapfel et al. 1998), may therefore be due to other external factors such as microbe-microbe interactions in the gut (Lebeer et al. 2008), adhesion factors (Greene and Klaenhammer 1994), and presence of nutrients and enzymes (Laparra and Sanz 2010), rather than to differences in pH.

The metabolic footprints provided a new possibility to study how NCFM is potentially influencing the conditions in the human gut. As expected, production of lactic acid was observed (Fig. 4b), which in vivo is expected to lower the pH and thereby protect the host from pathogenic infections (Sanders and Klaenhammer 2001). Additionally, lactic acid is believed to play role in cancer prevention (Hirayama and Rafter 2000). After 24 h of fermentation, also the amount of succinic acid was significantly increased (Table 3 and Fig. 4b). Succinic acid (Watanabe et al. 2010) could add to the positive effect of lactic acid in the intestines by lowering the pH value. We speculate, that production of succinic acid by NCFM is based on the glyoxylate cycle (Berg et al. 2001; Popov et al. 2005), as only a part of the citrate cycle was detected in the genome of NCFM (Altermann et al. 2005).

Adenosine was present in rather high amounts in all medium samples (M) and F3, whereas it was practically gone in F5 and F7 (Fig. 4a). The opposite was true for adenine (Fig. 4). It is not clear whether the only source for adenine production was adenosine, since a complete pathway for de novo synthesis of purines is present in genome of NCFM (Altermann et al. 2005). Survival of *Lactobacillus* in nutrient limited environments is reported to be dependent on the presence of an adenine source (Elli et al. 1999). We speculate that this is true also in the GIT. High amounts of adenine in F5 and F7, compared to F3, could be caused either by cell degradation or by extracellular metabolite production. Adenine may interact with intestinal epithelial cells, but this remains to be investigated. It may be speculated that free adenine from bacterial sources promotes epithelial cell metabolism.

The *P*-value between groups F5 and F7 (Table 3) suggest that arginine is the only metabolite found to be present in significantly different amounts, depending on the pH. No metabolic pathways were found for this compound to be synthesized by NCFM (Altermann et al. 2005) and most of the *Lactobacillus* species depend on external sources of arginine (De Angelis et al. 2002). It is therefore likely that the increase in arginine in F5 and F7 (Fig. 4a) originates from digestion of peptides. The difference between F5 and F7 (Table 3) might be caused by different activities of extracellular enzymes at pH 5 and 7.

After 24 h growth at pH 5 and 7, growth tests (Fig. 1b) and Live&Dead staining showed that dead cells were present in the medium. Therefore, one has to be aware that in the supernatant intracellular as well as extracellular metabolites might be present. It is important to differentiate between those two groups of metabolites because they play different roles. Thus, it has to be taken into consideration that released intracellular enzymes may act on components present in the medium. However, no significant differences were found between samples M3 and F3 (Table 3), where dead cells were also present. Therefore we find it likely that metabolites, found in higher concentrations in F5 and F7 than in M5 and M7 (Fig. 4), were extracellular.

## 5 Concluding remarks

In vitro tests coupled with metabolomic footprinting have shown that NCFM is increasing the concentration of lactic acid, succinic acid, adenine and arginine in the medium. Additionally, the strain consumed the adenosine present in the medium. The metabolomic approach has opened new possibilities to address how NCFM interacts in the GIT ecosystem through external metabolite production. The metabolism of NCFM did not change significantly between pH 5 and 7, suggesting that other environmental factors might have bigger impact on its colonization throughout the GIT.

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# Data in preparation

## Monocolonization with *Lactobacillus acidophilus* NCFM affects the intestinal metabolome as compared to germfree mice

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Data in preparation

# Monocolonization with *Lactobacillus acidophilus* NCFM affects the intestinal metabolome as compared to germfree mice (data in preparation)

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## Aim of the study

*Lactobacillus acidophilus* NCFM [1,2] colonization of germfree (GF) mice was carried out in order to map metabolites produced by the probiotic bacteria when growing in the intestinal environment, as well as host metabolites induced by the presence of the bacteria. *In vitro* experiments combined with the metabolomics methodology allow studies of metabolic mechanisms of NCFM's effect on the host throughout the gut environment. The use of a metabolomic approach in the area of microbial activity in the gut is completely new. Only a small handful of very recent studies address the host metabolome as a function of colonizing bacteria [3,4]. However, recent literature suggests that the intestinal microbiota influence not only the faecal metabolome, but also the metabolite profiles of e.g. in biofluids and specific host organs [5-8]. Therefore, many different mammalian samples were investigated for the effect of the NCFM strain, comparing the metabolome of monocolonized (MC) mice to GF mice, in selected parts of the gastrointestinal tract.

## Materials and methods

### *Lactobacillus acidophilus* NCFM inoculation preparation

*Lactobacillus acidophilus* NCFM was kindly provided by Danisco A/S. The strain was grown anaerobically at 37$^{o}$C for 24h in de Man, Rogosa and Sharpe (MRS) broth (Oxoid Ltd., Basingstoke, Hempshire, England). After centrifugation at 3000g for 15min, pellets were washed and resuspended in a sterile saline supplement with 0.1% peptone. Final concentration of the NCFM cells in the inoculum was around $6.3 \cdot 10^{9}$ CFU/ml.

### Animal handling

Animal experiments were conducted according to the Federation of European Laboratory Animal Science Associations (FELASA) and Danish legislation.

Swiss Webster mice, bred at the National Food Institute (DTU Food), were originally obtained from Taconic (Lille Skensved, Denmark) and kept in germfree isolators. Absence

of colonizing bacteria in germfree mice was confirmed by cultivation of fecal samples. A monocolonized (MC) group of 5 male mice at age of 5 weeks was colonized with 200µl of previously described NCFM inoculum, resulting in around $10^9$ cells per dosage. After the colonization, fecal samples from the MC mice were analyzed as described below in order to evaluate the efficiency of the NCFM colonization and stabilization in the GIT. A GF group of 5 male mice was also weaned at age of 5 weeks and kept germfree. Animals from both groups were terminated at age 8 weeks.

**Samples collection and metabolism quenching**

After utilization of the animals, blood was immediately taken by heart puncture, inserted in heparin tubes and centrifuged at 3000g for 15min, at 4°C. Plasma samples (supernatant) were frozen in the cryotubes using liquid nitrogen and kept at -80°C until further use.

Samples from the oral cavity (tongue), liver and internal parts (lumen) of the jejunum, caecum and colon were taken from all the mice. The mucus layer of jejunum and caecum was obtained by washing lumen-free intestinal parts in sterile MQ water and separated from the tissue, using cell-scrapers. All samples were frozen in the cryotubes using liquid nitrogen and kept at -80°C until further use. Luminal samples from MC mice, were analyzed as described below to calculate the number of NCFM cells throughout the intestines

**Enumeration of bacteria**

Fecal and luminal samples from jejunum, caecum and colon of the MC mice were suspended in sterile saline supplemented with 0.1% of peptone. NCFM were counted on MRS agar (Oxoid Ltd., Basingstoke, Hempshire, England) after anaerobic incubation at 37°C for 48h. Additionally, samples were screened for absence of contamination by plating on Luria-Bertani agar incubated at 37°C for 48h in an aerobic atmosphere.

**Metabolites extraction**

All samples were shipped on dry ice to Auckland University where metabolite extraction was performed (by me). Prior to each extraction method, samples were defrosted on ice and kept cold throughout the whole procedure.

Cold methanol and chloroform in a 1:1 ratio was added to the plasma samples in amounts proportional to the volume of the sample, meaning that 200µl of each solvent was added simultaneosly to the lowest plasma volume, increasing in amount accordingly to the sample volume. This ensured an equal proportion of water from plasma to methanol in each test. Tubes with the content were vortexed for 1 min and centrifuged at 4000g for 5min at 4°C. Methanol and chloroform extracts were stored separately at -20°C until further use.

Amounts of intestinal lumen and mucus were measured before each extraction. Samples were homogenized by grinding in the glass tubes kept on ice. 500µl of cold 80% methanol was added to the samples, vortexed for 1 min and centrifuged at 4000g for 5 min at 4°C. Methanol/water extracts were removed. This procedure was done 3 times, combining methanol/water extracts together for each sample. Afterwards, 500µl of cold chloroform was added to the sample, ultrasonificated for 1 min and centrifuged at 4000g for 5 min at 4°C. Chloroform extract was separated from the biomass and, as well as the methanol/water extracts, stored at -20°C until further use.

Weight of tissues (liver and part of the oral cavity) was measured before each extraction. Sample was homogenized by grinding in the glass tubes kept on ice. 2.5ml of cold 50% methanol and 2ml of chloroform was added to the sample.The mixtures were shaken for 1h on ice, stored at 4°C and centrifuged at 4000g for 5 min also at 4°C. A methanol/water extraction was subsequently performed twice. Finally, both extracts were separated from the biomass and stored at -20°C until further use.

Concentration of methanol in the polar extracts was decreased below 15%. Methanol/water extracts were freeze-dried and the chloroform ones dried under a nitrogen stream and kept at -20°C. Samples were secured with a silica gel to absorb the moist and shipped back to the Technical University of Denmark, LC-MS and DI-MS analyses were performed back in Denmark.

**Metabolite detection**

The methanol/water extracts were resolubilized in 5 % acetonitrile (Fluka, Sigma-Aldrich, Steinheim, Germany), ultrasonicated for 10 min at 4°C and centrifuged at 10000 g for 7 min at 4°C. The supernatant was removed and analysed by LCMS using a Dionex Ultimate 3000 RS liquid chromatigraph (Dionex, Germering, Germany) coupled to a Bruker maXis time of flight mass spectrometer equipped with an electrospray interphase (Bruker Daltonics, Bremen, Germany). Analytes were separated on a Kinetex pentafluorophenyl column 100 x 2.10 mm, 2.6 µm, 100Å (Phenomenex, USA), using the solvent system: A (5 mM ammonium formate with 0.1% formic acid; both from Fluka, Sigma-Aldrich, Steinheim, Germany), and B (acetonitrile, Fluka, Sigma-Aldrich, Steinheim, Germany, with 0.1 formic acid). Solvent programming was isocratic 0% B for 2 min followed by 5% at 5 min, then linear gradient up to 100% B at 10 min and 100% B at 12 min. The solvent composition was returned to initial conditions at 12.1 min and recalibrated to 14 min. Flow rate was 0.3 ml/min. The oven temperature was 40°C. Injection volumes were 3 µL. The following electrospray interphase settings were used: nebulizer pressure 2 bar, drying gas 10 L/min, 200°C, capillary voltage 4500V. Scan range was from 100 to 1000 m/z. Samples were analyzed in both positive and negative mode. External and internal calibration was done using sodium formate clusters (Sigma-Aldrich, Steinheim, Germany). Lock-mass calibration (hexakis(1H,1H,2H-perfluoroetoxy)phosphazene, Apollo Scientific, Stockport, UK) was applied in order to lower the measurement error to minimum.

Chloroform extracts were resolubilized in 80 % methanol (Fluka, Sigma-Aldrich, Steinheim, Germany) with 0.1 % (vol/vol) formic acid, ultrasonicated for 10 min at 4°C and centrifuged at 10000g for 7 min at 4°C. The supernatant was removed and analysed by DI-MS, as follows: 1 µl was injected into 0.25 ml/min flow of 80 % acetonitrile, which was introduced directly into the electrospray ionsource. The mass spectrometer with the electrospray interphase settings were the same as described before. Scan range was from 100 to 1000 m/z. Data was acquired for 2 min for each sample. Samples were analyzed in both positive and negative mode. External calibration was done using sodium formate clusters (Sigma-Aldrich, Steinheim, Germany).

**Multivariate data analysis**

The differences in metabolite profiles were evaluated by principal component analysis (PCA). LC-MS data in negative was grouped into buckets according to the mass to charge ratio (*m/z*; Da) and retention time (RT; min). Size of each bucket was 1 Da and 1 min, from 0.5-5 min and *m/z* 100-1000 Da. Data was mean centred and normalised by the sum of bucket. PCA models were calculated using Profile Analysis 2.0 (Brucker Daltonics, Bremen, Germany).

## Preliminary results

During 3 weeks of incubation, numbers of *Lactobacillus acidophilus* NCFM in the MC mice were stable, approximately $10^9$ CFU/g of faeces (Figure 1). Luminal samples differed in the number of bacteria, gradually increasing from the jejunum to colon (Figure 2).

Studies focussed on the full metabolome, extracting polar and non-polar metabolites. Initial PCA analysis of the LC-MS data in negative mode showed a distinct difference between most of the samples from GF and MC mice (Figures 3, 4, 5B, 6, 7), indicating that the NCFM strain had an impact on the region-dependent metabolome of the mammalian host as well as global one (liver and plasma samples; Figs. 6 and 3). However, mucus samples from the jejunum did not show any grouping of the data (Figure 5A). This may be because the amount of biomass from the jejunal mucus available for the metabolite extraction was very low. This could have caused lack of separation between the groups, at least in the negative mode of LC-MS.

Data buckets for the luminal samples showed significant similarities in the metabolic profile of caecum and colon (Figures 4B and 4C). The same metabolites seemed to be causing the group separations in both cases for the LC-MS data in negative mode. Similarly, numbers of bacteria in the lumen from caecum and colon were very similar (Figure 2) in the MC mice. Some of the buckets were present in all of the luminal samples (Figure 4).

## Preliminary conclusions and remaining work

Preliminary studies showed a good indication of the influence of the NCFM strain on the metabolome of the host. However, PCA analyses of the LC-MS data in positive mode, DI-MS data in both, positive and negative modes are still required. After selecting the buckets responsible for the group separation, P-values for selected buckets will be calculated. Metabolite identification and confirmation will be done for compounds with P-values lower than 0.05, comparing intensities from GM and MC samples.

## Acknowledgments

This work was supported by a grant from the Øresund Food Network (co-financed by The European Regional Development Fund and Vækstforum Hovedstaden; grant no.09198-02 ‘‘Bacterial Impact on the Gut Metabolome’’) and by the Danish Strategic Research Council’s Committee on Food and Health (FøSu; project no. 2101-06-0067 ‘‘Biological Production of Dietary Fibres and Prebiotics’’). Authors thank Kate Vina Vibefeldt, Bodil Madsen and Dang-Dung Nguyen for excellent technical support.

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## Figures

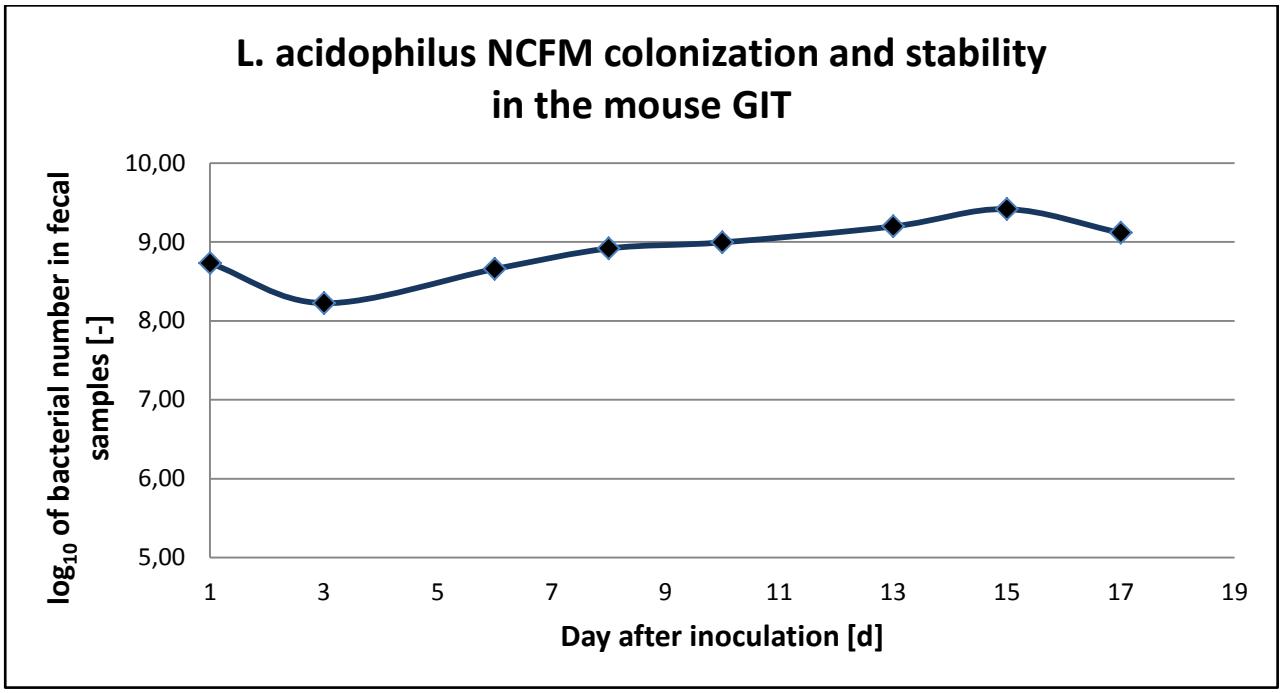



**Figure 1** NCFM number as $log_{10}$CFU in faecal samples of MC mice.

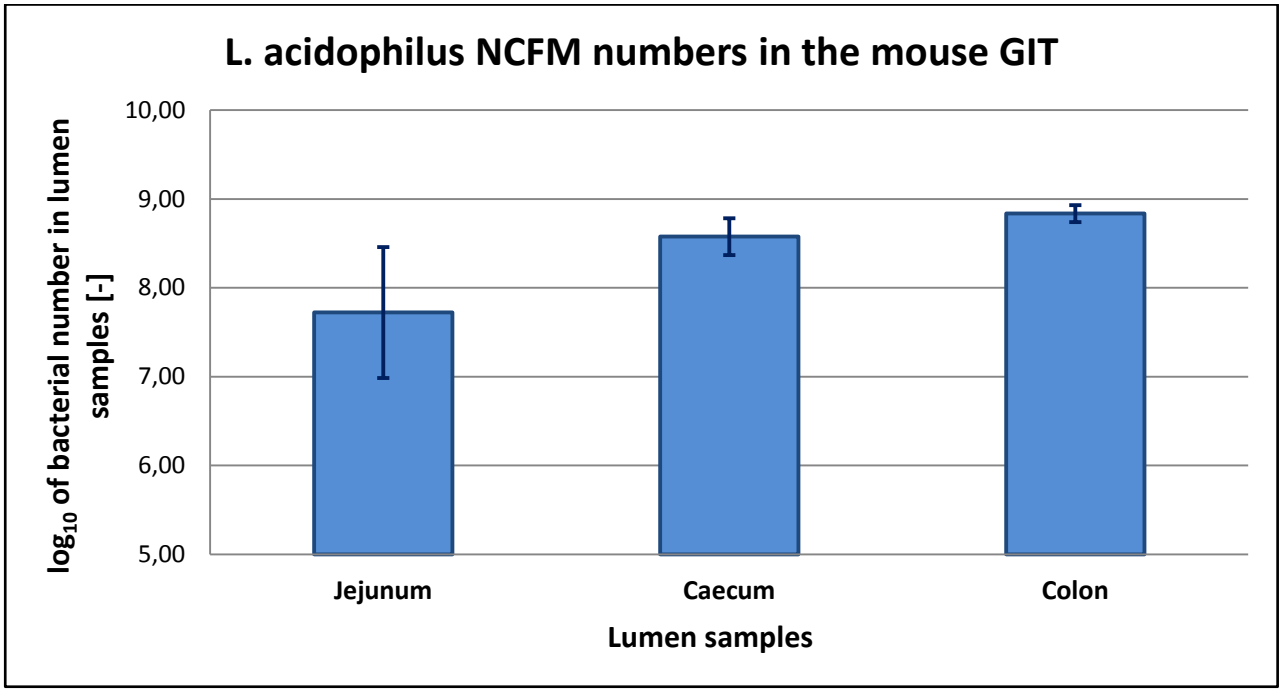



**Figure 2** NCFM number as $log_{10}$CFU in luminal samples from jejunum, caecum and colon of MC mice.

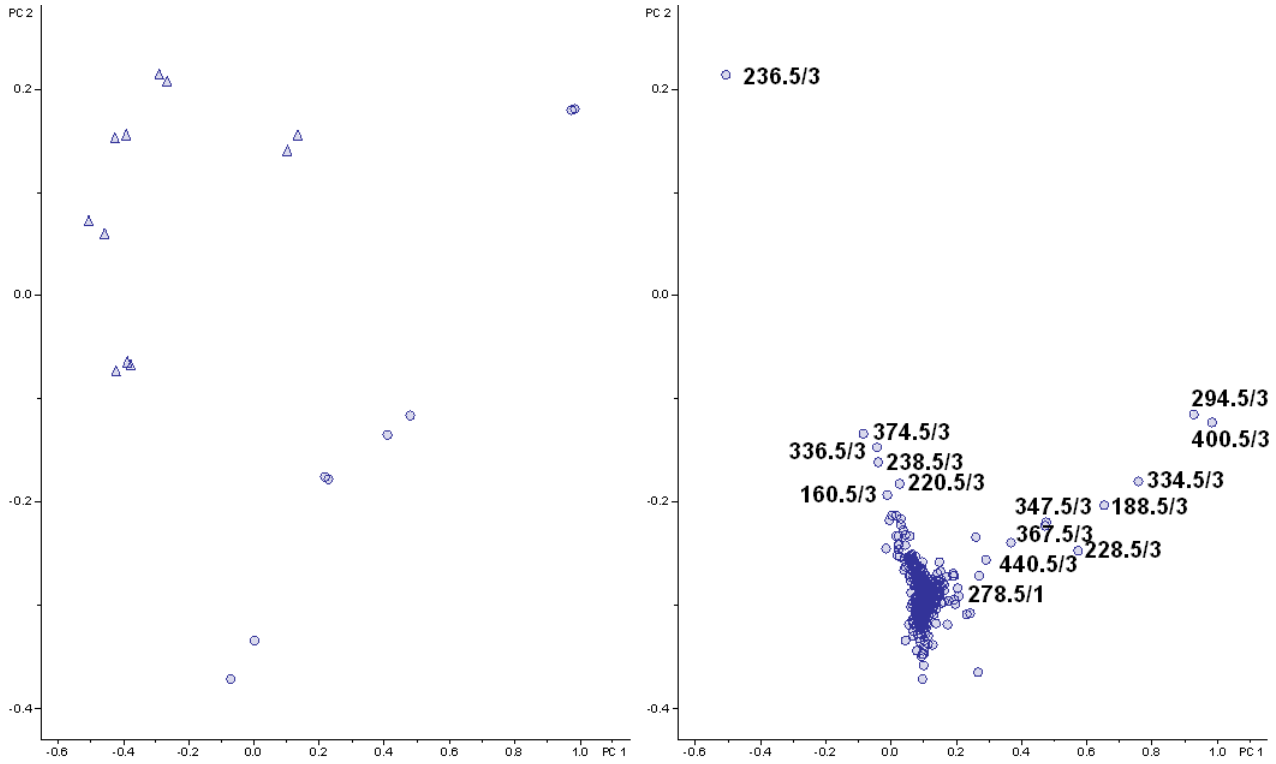
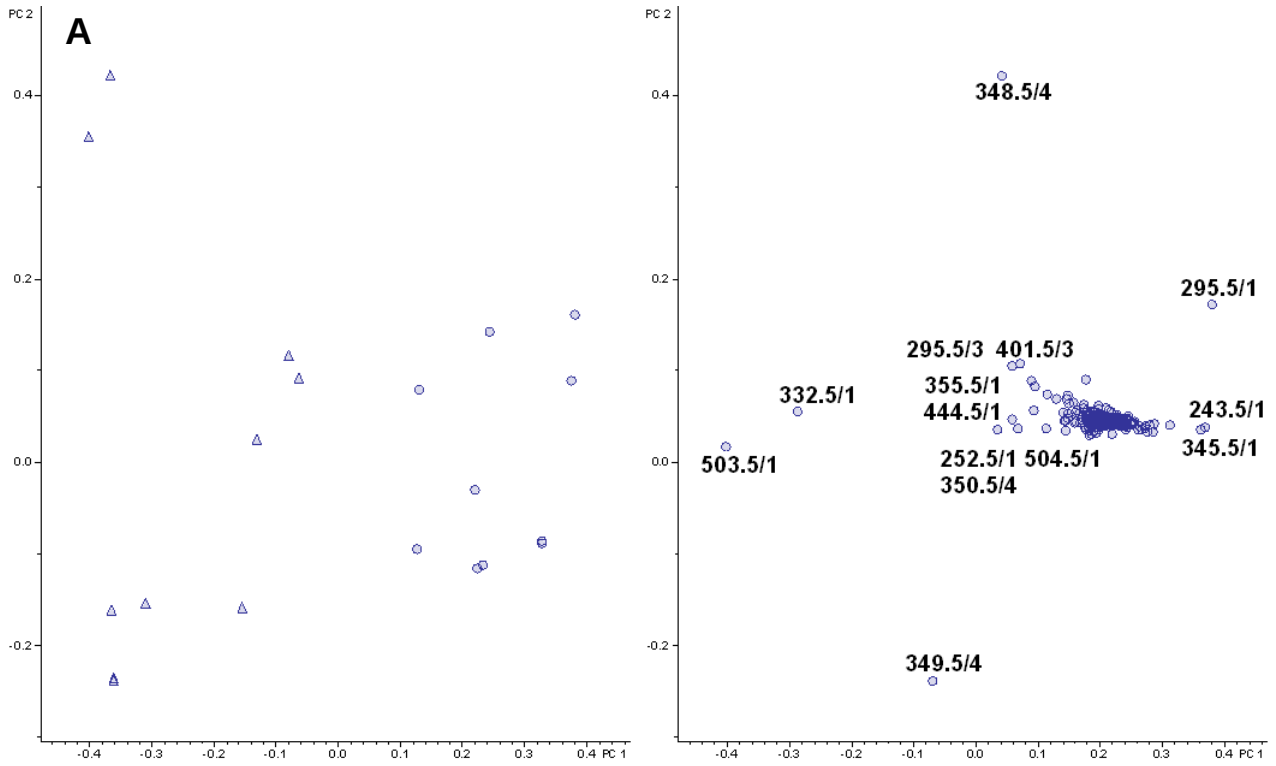



**Figure 3** PCA score (left) and loading (right) plot of the LCMS data in negative mode. Presented on the score plot plasma samples from GF (∆) and MC (o) mice. Numbers on the loading plot refer to the data bucket as mass to charge ratio (*m/z;* Da) per retention time (RT; min).



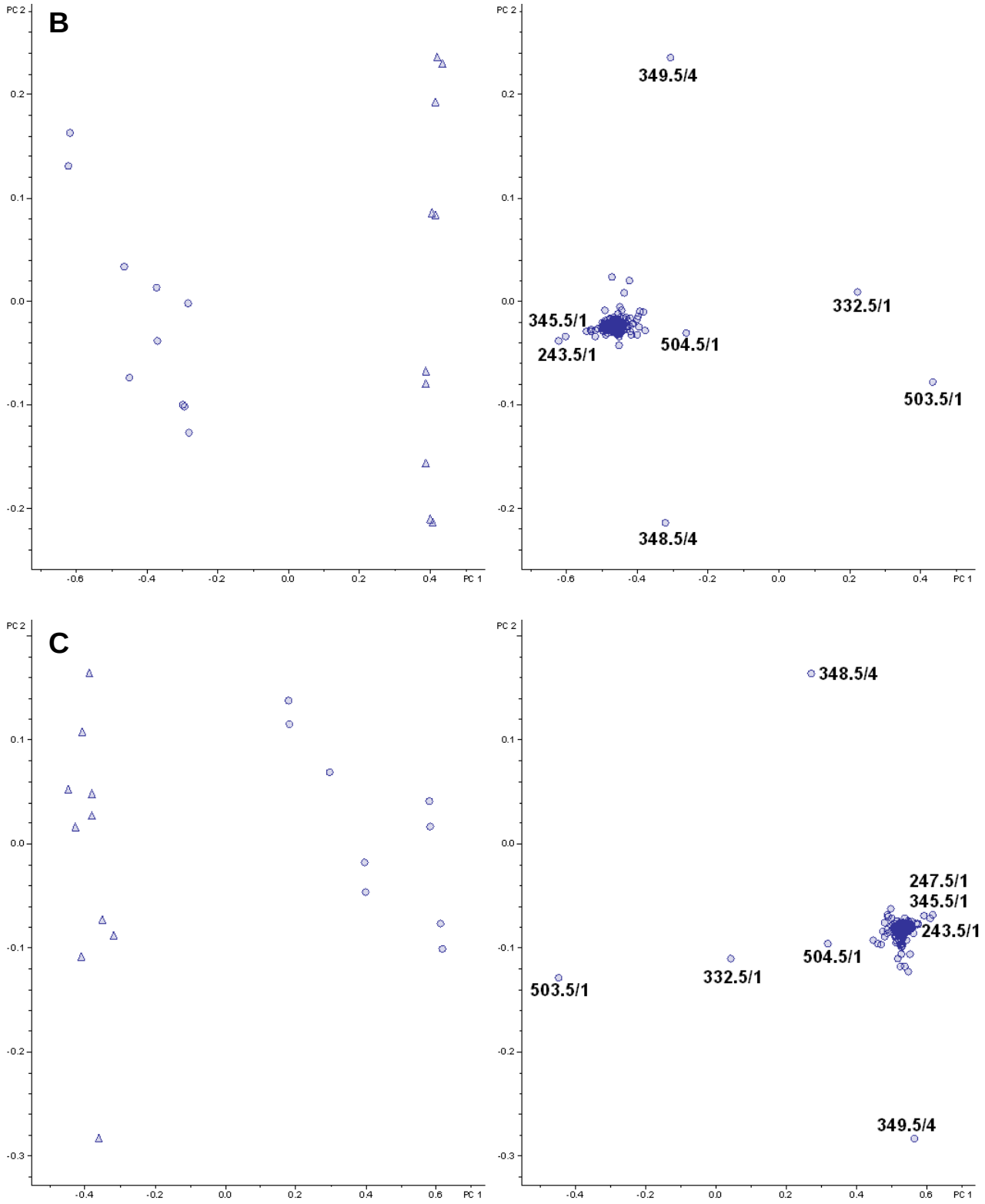



**Figure 4** PCA score (left) and loading (right) plot of the LCMS data in negative mode. Presented on the score plots lumen samples from jejunum (A), caecum (B) and colon (C) of GF (Δ) and MC (o) mice. Numbers on the loading plot refer to the data bucket as mass to charge ratio (*m/z;* Da) per retention time (RT; min).

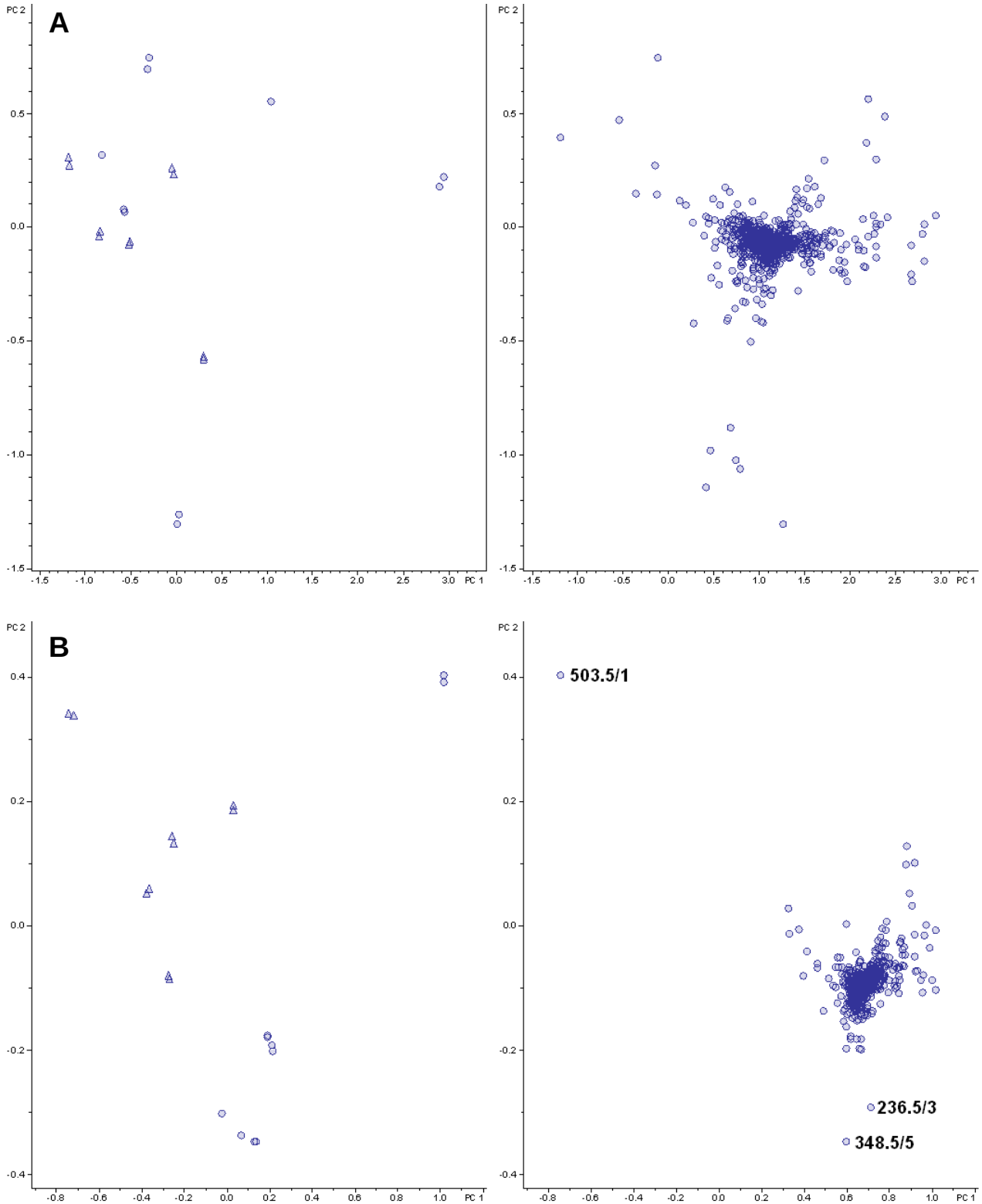



**Figure 5** PCA score (left) and loading (right) plot of the LCMS data in negative mode. Presented on the score plots mucus samples from jejunum (A) and caecum (B) of GF (∆) and MC (o) mice. Numbers on the loading plot refer to the data bucket as mass to charge ratio (*m/z;* Da) per retention time (RT; min).

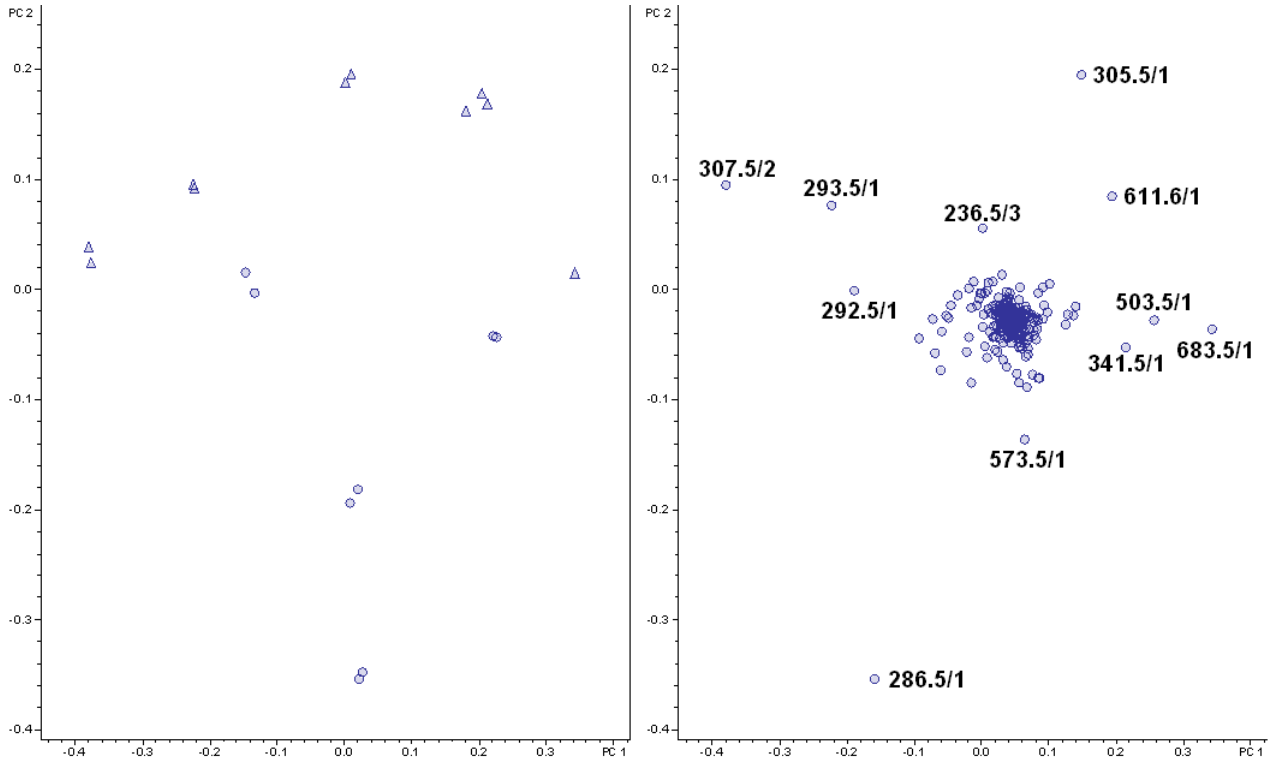



**Figure 6** PCA score (left) and loading (right) plot of the LCMS data in negative mode. Presented on the score plot liver samples from GF (∆) and MC (o) mice. Numbers on the loading plot refer to the data bucket as mass to charge ratio (*m/z;* Da) per retention time (RT; min).

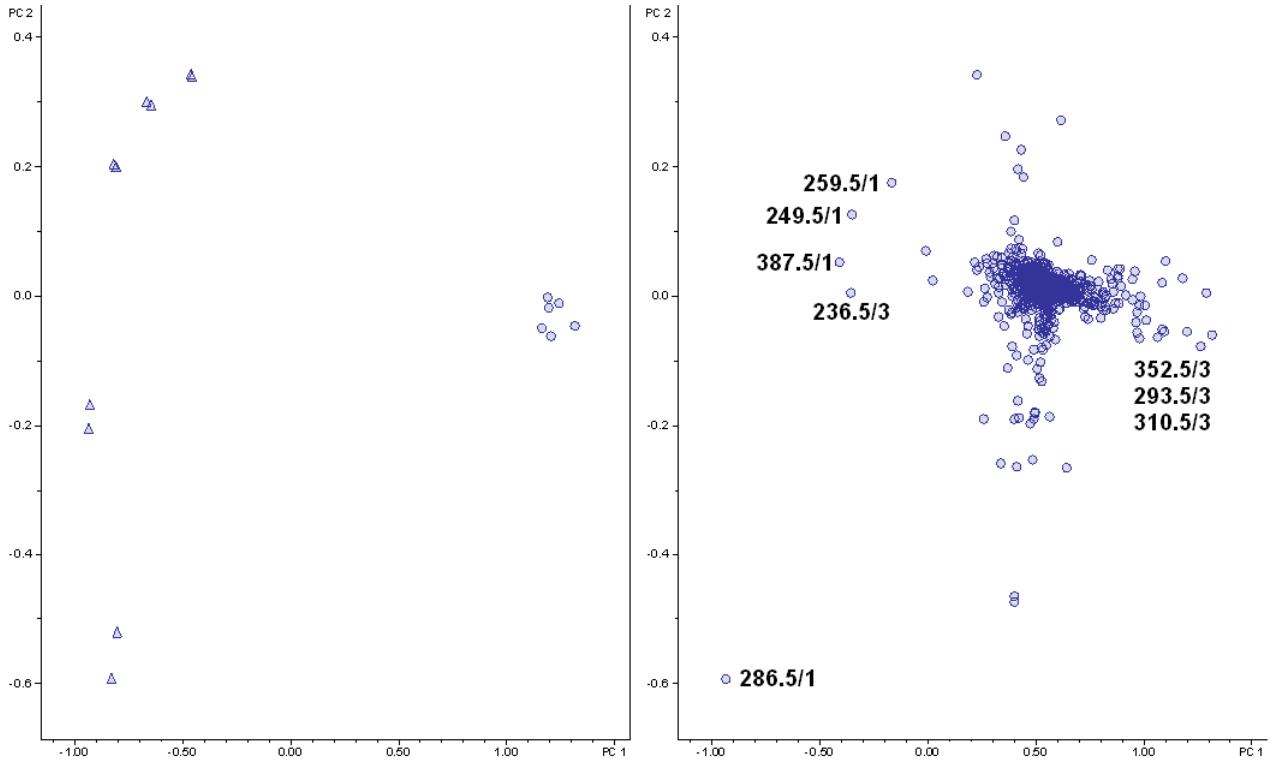



**Figure 7** PCA score (left) and loading (right) plot of the LCMS data in negative mode. Presented on the score plot oral cavity samples from GF (∆) and MC (o) mice. Numbers on the loading plot refer to the data bucket as mass to charge ratio (*m/z;* Da) per retention time (RT; min).

# Manuscript I

## A combined metabolomic and phylogenetic study reveals putatively prebiotic effects of high molecular weight arabino-oligosaccharides when assessed by *in vitro* fermentation in bacterial communities derived from humans.

**Sulek, K.**; Vigsnaes, L.K.; Schmidt, L.R.; Holck, J.; Frandsen, H.L.; Smedsgaard, J.; Skov, T.H.; Meyer, A.S.; Licht, T.R.

Ready for submission

# A combined metabolomic and phylogenetic study reveals putatively prebiotic effects of high molecular weight arabino-oligosaccharides when assessed by *in vitro* fermentation in bacterial communities derived from humans.

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## Abstract

Prebiotic oligosaccharides are defined by their selective stimulation of growth and/or activity of bacteria in the digestive system in ways claimed to be beneficial for health. However, apart from the generation of short chain fatty acids, little is known about bacterial metabolites created by prebiotic fermentation, and the impact of the size (chain length) of the oligosaccharides remains largely unstudied.

We carried out *in vitro* fermentations in human fecal microbial communities (derived from six different individuals), using high-mass (HA, >1kDa), low-mass (LA, <1kDa) and mixed (BA) sugar beet arabino-oligosaccharides (AOS), respectively, as carbohydrate sources, and including fructo-oligosaccharides (FOS) as control. Changes in the bacterial communities and the metabolites produced were analyzed by qPCR and LC-MS, respectively.

Microbial response to the carbohydrates was highly dependent on the individual microbial ecosystem. All tested carbohydrate sources resulted in a significant increase of *Bifidobacterium* spp. between 1.79 fold (HA) and 1.64 fold (FOS) in the microbial populations after fermentation. Additionally, HA and FOS fermentation caused a decrease in levels of *Desulfovibrio* spp.

HA caused the highest increase of metabolites putatively beneficial to human gastrointestinal health. Fermentation of AOS, and in particular of HA, additionally resulted in an increase of various bacterial metabolites that may potentially be involved in biosynthetic pathways of epithelial cells when produced in vivo in the gut. In all six fecal communities, the HA fraction gave a metabolic response that was more similar to the established prebiotic FOS than seen for the LA and BA fractions.

## Introduction

The human gastrointestinal tract (GIT) produces a large amount of enzymes capable of hydrolyzation of various disaccharides and a few specific polysaccharides (starches). However, most complex oligo- and polysaccharides including e.g. arabino- and fructo-oligosaccharides cannot be degraded by the human digestive enzymes. These substrates may instead be metabolized by the very diverse ecosystem of bacteria inhabiting the human gut [1].

Prebiotic oligosaccharides are non-digestible oligosaccharides defined by their selective stimulation of growth and/or activity of bacteria in the digestive system in ways claimed to be beneficial for health [2].

Inter-bacterial interactions, as well as interactions between bacteria and host are based on a variety of mechanisms. Biochemical messages can be sent by simple or complex abiotic molecules as well as by genetic sequences [3]. Previous studies of prebiotic degradation were mainly focused on bacterial production and epithelial absorption of short chain fatty acids (SCFA) [4-6]. A more exhaustive approach is metabolomic footprinting, which describes the bacterial exometabolome, defined as the pool of molecules excreted by a bacterial community into the surroundings [7]. Such molecules are likely to be involved in signaling between bacteria or to the host. Metabolomics has previously been found useful in studies of the intestinal microbial ecosystem [8,9]

The aim of the present study was to evaluate the potential prebiotic effect of sugar beet arabino-oligosaccharides (AOS) in comparison to the established prebiotic fructo-oligosaccharide (FOS) [10,11]. We addressed whether *in vitro* fermentation of differently sized AOS molecules caused different changes in intestinal bacterial communities isolated from six healthy humans. Additionally, the putatively prebiotic (health-promoting) effect of AOS was addressed using a new approach: Metabolomics.

## Materials and Methods

### Arabino-oligosaccharide substrates

Sugar beet arabino-oligosaccharides (AOS) were obtained from Danisco A/S (Nakskov, Denmark). The arabino-oligosaccharides were derived from a liquid side stream from the ultrafiltration and diafiltration step in the sequential acid extraction of pectin with nitric acid from sugar beet pulp, involving removal of insoluble cellulose, ultrafiltration, and diafiltration with a 50 kDa cutoff [12]. The pulp was dried prior to extraction.

Separation of arabino-oligosaccharides from the base solution of arabino-oligosaccharides (BA) according to size was performed in a 200mL stirred membrane reactor model 8200 (Millipore, Billerica, MA) equipped with a 1 kDa MWCO regenerated cellulose membrane (Millipore, Billerica, MA) connected to compressed nitrogen for flux regulation. Filtration was performed at room temperature. Filtration was performed at 3 bar until the retentate volume was 30% of the sample volume, and followed by diafiltration in one sample volume of deionized water. The permeates enriched in low molecular weight oligosaccharides were denoted LA and the retentate enriched in high molecular weight oligosaccharides were denoted HA. Free sugar content and monosaccharide composition was determined by acid hydrolysis and HPAEC as described previously [11].

### Size exclusion

HPSEC was performed using a P680 HPLC pump, an ASI-100 automated sample injector, and an RI-101 refractive index detector (Dionex Corp., Sunnyvale, CA). Samples were separated on a Shodex SB-806HQGPCcolumn (300 x 8mm) with a Shodex SB-G guard column (50 x 6 mm) from Showa Denko K.K. (Tokyo, Japan) with 100 mM sodium acetate pH 6 as mobile phase used with a flow rate of 0.5 mL/min. Temperature was maintained at 40 °C. Data were collected and analyzed with the program Chromeleon 6.80 SP4 Build 2361 software (Dionex Corp., Sunnyvale, CA).

**Removal of monosaccharides from the Semi Synthetic Substrates**

In order to reduce the amount of monosaccharides present in the AOS-based substrates, we carried out an initial bacterial fermentation using *L. acidophilus* NCFM. This strain was kindly provided by Danisco A/S and chosen because we have previously mapped the metabolites consumed and produced by this strain [13], and because it's genome sequence [14] does not contain the enzymes needed for AOS degradation.
*L. acidophilus* colonies were grown anaerobically at 37$^{o}$C overnight in MRS broth (Oxoid Ltd., Basingstoke, Hempshire, England) and dissolved $10^{7}$ times into Semi Synthetic Medium (SSM) [15] containing 1% glucose. After 7 hours of resulting in $10^{5.8}$ CFU/ml of L. acidophilus NCFM, the cultures were diluted 100 fold into SSM containing either 1% glucose (control), 20 g/L of BA, 20g/L LA, or 20g/L HA. After 24h of anaerobic incubation, removing non-arabinan monosaccharides from the media, the cultures were centrifuged at 3000g for 5min at 4°C, whereafter, supernatants were sterile filtrated in order to remove remaining *L. acipdophilus* cells and kept in at 4°C until further use. The final concentration of the arabinan oligosaccharides was estimated to 10 g/L.

**Subjects and fecal sample collection**

Fecal samples were obtained from six healthy volunteers (four women and two men). None of the participants had been treated with antibiotics for at least 3 months before enrolment and had no history of gastrointestinal disorder. The mean age of the participants was 41±9 years. The samples were collected in airtight containers at home by the participants and stored at 4°C (limited storage time was encouraged [16]) until delivery to the laboratory, where they were processed immediately. The fecal samples were homogenized in 50 % glycerol (1:1 dilution) in an anaerobic cabinet (Macs Work Station, Don Whitley) containing 10% $H_2$, 10% $CO_2$, and 80% $N_2$, and stored at -80°C until further analysis, as described below.

**In vitro fermentation by human fecal bacterial communities**

Fermentation studies were carried out to assess the effect of BA, LA and HA on the microbial composition and activity in human fecal samples, while parallel incubations with the established bifidogenic substrate FOS [17] and no carbohydrates, respectively, were used as controls. Fecal samples prepared as described above were defrosted in an anaerobic cabinet and 10% (w/v) fecal slurry was prepared by mixing the samples with anoxic PBS (Oxoid, Greve, Denmark) immediately before fermentation.

Sterile SSM supernatants prepared as described above were mixed 1:1 with sterile minimal basal medium containing 2 g/L of peptone water (Oxoid Ltd., Basingstoke, Hempshire, England), 1 g/L of yeast extract (Sigma Chemical co., St. Louis, Missouri, USA), 0.1 g/L of NaCl (Merck KGaA, Darmstadt, Germany), 0.04 g/L of $K_2HPO_4$ (Merck KGaA, Darmstadt, Germany), 0.04 g/L of $KH_2PO_4$ (Merck KGaA, Darmstadt, Germany), 0.01 g/L of $MgSO_4 \cdot 7H_2O$ (Merck KGaA, Darmstadt, Germany), 0.01 g/L of $CaCl_2 \cdot 2H_2O$ (Merck KGaA, Darmstadt, Germany), 2 g/L of $NaHCO_3$ (Merck KGaA, Darmstadt, Germany), 0.5 g/L of L-cysteine hydrochloride (Sigma Chemical co., St. Louis, Missouri, USA), 50 mg/L of hemin (Sigma Chemical co., St. Louis, Missouri, USA), 10 µl/L of vitamin K1 (Sigma Chemical co., St. Louis, Missouri, USA), 0.05 g/L manganese sulfate monohydrate ( Merck KGaA, Darmstadt, Germany) and 1 mL/L of Tween 80 (VWR, Darmstadt, Germany). The pH of the final solution was adjusted to 7. Estimated (not accurate) concentrations of AOS were 5 g/L. Positive controls were made by adding 5 g/L of FOS to the SSM supernatant prepared by NCFM fermentation of glucose, and negative controls by adding nothing to the same SSM supernatant. All solutions were reduced over night in an anaerobic cabinet and inoculated with fecal slurry prepared as described above to a final concentration of 1% feces. Tube caps were loosely placed on the vials to allow gas exchange but avoid evaporation. Each fermentation was carried out in triplicates for each fecal community, carbohydrate source and controls. The fermentation was non-pH controlled and non-stirred due to the low reaction volume (6-7 mL) and was carried out in an anaerobic cabinet at 37°C. At the beginning of the fermentation (time 0), and after 24 hours (time 24), 1 ml samples were taken and centrifuged at 3000 g for 15 min at 4°C. The supernatants were used for metabolite profile analysis and the pellets were used for extraction of bacterial DNA as described below.

**Extraction of bacterial DNA**

DNA was extracted from each of the triplicate fermentation samples using the QIAamp DNA Stool mini kit (Qiagen, Hilden, Germany) with preceeding bead beating as previously described [18]. The concentration of the purified DNA was measured by Qubit® 2.0 Fluorometer (Invitrogen) and the DNA was stored at -20°C until use.

**Real-Time PCR assay**

Amplification and detection of purified bacterial DNA by Real-time PCR was performed with the ABI Prism 7900 HT from Applied Biosystems using optical grade 384-well plates. Each amplification reaction was done in duplicate for each of the triplicate fermentation samples in a final volume of 11 µl containing; 5.50 µl SYBR® Green Master Mix (Applied Biosystems, Denmark), 200 nM of each of the primers (Eurofins MWG Synthesis GmbH, Ebersberg, Germany), 2 µl template DNA (1 ng/µL), and Nuclease-free water purified for PCR (Qiagen). The amplification program comprised one cycle at 50°C for 2 min; one cycle at 95°C for 10 min; 40 cycles at 95°C for 15 sec.; 60°C for 1 min, and finally one cycle of melting curve analysis for amplicon specificity at 95°C for 15 sec, 60°C for 20 sec. and increasing ramp rate by 1.92°C/min until 95°C for 15 sec. The qPCR data was baseline corrected and $N_0$-values, representing initial concentrations of the specified 16S rRNA genes, were calculated using the LinRegPCR software (version 11.1, based on Ruijter *et al.* [19]). All results were calculated as means of duplicate $N_0$ estimations, equal values required. The relative quantities of gene targets encoding 16S rRNA sequences of the bacterial taxa were calculated using $N_0$ (bacterial target)/$N_0$ (total bacterial population). The applied specific 16S rRNA-targeting primers are listed in Table S1 (supplementary data). Prior to quantification, all primers were tested to confirm sensitivity and specificity using DNA from pure bacterial species (A. Bergström, T.R. Licht, A. Wilcks, J. B. Andersen, L. R. Schmidt, H. Grønlund, L. K. Vigsnæs, K. F. Michaelsen and M.I. Bahl, in press)

**Metabolism quenching**

Time 0 and time 24 supernatants from the fermentations were quickly transferred into cold methanol (Fluka, Sigma-Aldrich, Steinheim, Germany, stored at -80°C) in the ratio 1:1 to quench the metabolism. Samples were stored at -80°C and centrifuged at 15000g for 5 min at 4°C just before LCMS (Liqiud Chormatography – Mass Spectrometry) analysis was carried out as described below.

**Metabolite detection by LCMS**

Metabolite analysis was conducted using a Dionex Ultimate 3000 RS liquid chromatograph (Dionex, Germering, Germany) coupled to a Bruker maXis time of flight mass spectrometer equipped with an electrospray interphase (Bruker Daltonics, Bremen, Germany). Analytes were separated on a Kinetex pentafluorophenyl column 50 x 2.10 mm, 2.6 µm, 100Å (Phenomenex, USA), using the solvent system: A (10 mM ammonium formate at pH 3.5), and B (acetonitrile). Solvent programming was isocratic 0% B at 0 min followed by a linear gradient up to 100% B at 7 min and 100% B at 8 min. Flow rate was 0.25 mL/min at 0 min, and increased to 0.4 mL/min at 7 min. Solvent composition and flow were returned to initial conditions at 8.2 min. The oven temperature was 40°C. Injection volumes were 1 µL. The following electrospray interphase settings were used: Nebulizer pressure 2 bar, drying gas 10 L/min, 200°C, capillary voltage 4000V. Scan range was from 50 to 800 m/z. Samples were analyzed in both positive and negative mode. External and internal calibration was done using sodium formate clusters (Sigma-Aldrich, Steinheim, Germany). Lock-mass calibration (hexakis(1H,1H,2H-perfluoroetoxy)phosphazene, Apollo Scientific, Stockport, UK) was applied in order to lower the measurement error to minimum. MSMS fragmentation of the selected masses (Tables 3 and 4) was done with a collision energy at 14 eV at mass 100 ramped lineary to 20 eV at mass 500 and to 30 eV at mass 1000.

## Metabolite identification

Metabolite identification was based on the exact mass to charge ratio (*m/z*) with a very low measurement error, MSMS fragments (Tables 3 and 4), metabolites found in the Human Metabolome Database (HMDB; [20]) and the Metabolite Mass Spectral Database (METLIN; [21]) where MSMS spectra of some selected metabolites are present, and references to the bacterial metabolism presented in the discussion part of this paper.

## Statistical analysis of the PCR data

Statistical analysis of the qPCR data was performed with the GraphPad Prism software (version 5.03; GraphPad Software Inc., La Jolla, CA). One-way analysis of variance and Tukey's multiple comparison tests were used to determine significant differences in the density of selected bacterial taxa in the different fermentations (NC, FOS, BA, LA and HA). Homogeneity of variance was assessed using Bartlett's test for equal variances. Log-transformations were performed before statistical analysis of qPCR measurements that did not meet this criterion. The nonparametric Kruskal-Wallis test and Dunn's multiple comparison tests were used for datasets, which did not have homogeneity of variance even after log-transformation. Tests were considered statistically significant when P-values lower than 0.05 were obtained.

## Multivariate data analysis

### *PCA analysis*

LCMS data were grouped into buckets of 1 min and 1 m/z differences in the range from 0.5 to 9 min and 50 to 800 m/z and normalized by the sum of buckets in the analysis by use of Profile Analysis 2.0 (Brucker Daltonics, Bremen, Germany).

The next step of the data analysis was done in Excel. A set of equations, presented below, was used to select metabolites present in the medium after 24h and produced exclusively due to the addition of the given carbohydrate.

First, the metabolites which were present already before the fermentation ($CH_0$), were subtracted from the metabolites present after 24 hours of fermentation ($CH_{24}$), and the remaining metabolites were represented as an average value CH(A) of intensities of the given bucket in three independent fermentations (I, II and III):

$CH_{24}(I)- CH_0(I) = CH(I)$; if $CH(I) \leq 0$, then $CH(I) = 0$; $(CH(I) + CH(II) + CH(III))/3 = CH(A)$

Secondly, the average amount of specific metabolites NC(A) produced only due to metabolism of the basal medium were identified by analysis of the control incubations (NC) carried out without addition of a carbohydrate source:

$NC_{24}(I)- NC_0(I) = NC(I)$; if $NC(I) \leq 0$, then $NC(I) = 0$; $(NC(I) + NC(II) + NC(III))/3 = NC(A)$

Finally, the metabolite values M(A) to be included in the Principal Component Analysis (PCA) were calculated as: $CH(A) – NC(A) = M(A)$; If $M(A) \leq 0$, then $M(A) = 0$

P-values describing differences between the experiments carried out with different substrates were calculated by t-test. PCA plots made in LatentiX 2.10 were based on the metabolite buckets showing significant differences between the tested carbohydrate types (P-value < 0.05; data not shown). Data were mean centered to avoid the influence of LCMS noise on the model and normalized (2-norm). From the positive mode, due to the presence of TWEEN in the medium, which was necessary for growth of lactobacilli, all m/z buckets at 3 min, 4 min and 7 min were removed. From the negative mode buckets with mass 555.5, 665.5, 666.5 (lock mass), 187.5, 188.5 and 189.5 at all time buckets were disregarded due to the noise they were creating.

Selected metabolites from previous PCA plots were used together with the PCR data (before log-transformation) to create a PCA plot (Figure 6) in LatentiX. Data was autoscaled and normalized (2-norm).

**Statistical analysis of the LCMS data**

Heat maps were created to illustrate the P-values of differences between levels of selected metabolites present before and after fermentation, taking into consideration only metabolites which increased during fermentation. P-values were calculated using t-test in Excel. Data used in this analysis were normalized by the sum of buckets by use of Profile Analysis 2.0.

## Results

### Oligosaccharide composition

Size exclusion chromatography (Figure 1) showed that the base solution of arabino-oligosaccharides (BA) had a dual distribution with one peak at 24.7 minutes corresponding to monomers and a larger broader peak around 1.0 kDa corresponding to the oligosaccharides. The low molecular weight fraction (LA) showed a similar profile, but with a tendency towards a lower content of oligosaccharides below 1.0 kDa. The high molecular weight fraction (HA) showed one homogeneous peak around 1.0 kDa with only a minor peak at 24.7 minutes indicating that the monosaccharide content was significantly reduced, but not removed completely. The BA substrate contained relatively high amounts of monosaccharides, mainly glucose (13.3% w/w), arabinose (10.1% w/w) and fructose (8.0% w/w). Other pectin derived free sugars like rhamnose, galacturonic acid, galactose, and fructose was found in minor amounts (2.3% w/w in total). Acid hydrolysis revealed that the residual 66.3% of the substrate was comprised of arabino-oligosaccharide moieties. The relative high content of monosaccharides compared to analysis of similar substrates [11,20] might be due to the drying of the pulp prior to the acidic extraction. LCMS analysis of the BA fraction revealed several peaks besides those identified by comparison to linear arabino-oligosaccharides, which indicated that the arabino-oligosaccharides applied in this study to a large extent were branched (data not shown). Initial fermentation with *Lactobacillus acidophilus* NCFM removed a significant amount of the non-arabinan monosaccharides present in the arabino-oligosaccharides fractions, as detected by LCMS (data not shown).

### Real-Time PCR studies of bacterial community composition

After fermentation in fecal slurries obtained from six different healthy subjects, quantitative PCR was applied to measure the density of gene targets encoding 16S rRNA of selected bacterial taxonomic units. The ability of the substrates to selectively stimulate the growth of a given bacterial taxon was compared to that of the NC (No added Carbohydrate)

fermentations (Table 1 and Figure S1). The fecal communities fermented on BA, LA and HA selectively increased the relative abundance of *Bifidobacterium* spp. significantly ($P<0.01$, $P<0.001$, and $P<0.001$, respectively) when compared to the NC fermentations. The densities of bifidobacteria after fermentation of BA, LA and HA were however not significantly different from the densities obtained by fermentation of FOS, which is considered to be the "golden standard" within the field of prebiotics. Also the densities of *Lactobacillus* spp. were higher in the BA, LA, HA and FOS fermentations than in the NC samples, although this was not statistically significant ($P=0.18$, $P=0.21$, $P=0.16$ and $P=0.23$, respectively). The relative abundances of the *C. coccoides* group and *Alistipes* spp. were significantly lower in fecal communities fermented on LA than in the NC fermentations ($P<0.05$ and $P<0.05$, respectively). FOS fermentation resulted in a significantly lower relative abundance of *Alistipes* spp. and *Desulfovibrio* spp. than the NC fermentations ($P<0.05$ and $P<0.05$, respectively). A significantly lower density of *Desulfovibrio* spp. was additionally observed in the fecal communities fermented on HA ($P<0.01$). No statistical difference in bacterial density after fermentation of the four different substrates was seen for the remaining investigated bacterial taxa.

Major individual differences between intestinal bacterial ecosystems derived from different subjects were expected to occur. The density of 16S rRNA genes of four different bacterial taxonomic units, showing alteration after fermentation (*Bifidobacterium* spp., *C. coccoides* group, *Alistipes* spp. and *Desulfovibrio* spp.), were thus determined for each of the six individual fecal communities (Table 2). Substantial individual differences were observed depending on substrate and bacterial target. However, fermentations in all of the six intestinal communities resulted in significantly higher increases of bifidobacteria than measured in the NC samples. When comparing of the amount of increase caused by the three substrates (BA, LA and HA), it varied significantly among the six fecal community. FOS fermentation resulted in significantly lower fold changes of bifidobacteria in three out of the six fecal communities than seen for the arabino-oligosaccharide based substrates. Only one fecal community (derived from individual 2) showed no significant difference in bifidobacterial increase caused by the tested substrates. The densities of the *C. coccoides* group were either unaltered or significantly lower after fermentation either of the four substrates. However, fermentation of FOS generally caused less decrease of *C. coccoides* than observed for BA, LA and HA. In all six bacterial communities, densities of *Alistipes*

spp. and *Desulfovibrio* spp. were either unaltered or significantly lower after fermentation of either of the four substrates, however the level of decrease varied between communities.

**Metabolomic studies**

After quenching the metabolism, LCMS analysis of samples taken before and after fermentation was done in positive and negative mode. Only buckets where significant differences were observed between at least 2 substrates were included in the further analysis, which significantly reduced the amount of variables (metabolites). PCA analysis of metabolite data (Figures S2 and S3) from positive mode showed, that lack of metabolite profile clustering was clearly affected by bacterial source communities and that differentiation caused by fermentation substrate was therefore unclear. However, in the negative mode, FOS-fermentation samples clustered separately from all three types of arabino-oligosaccharides (Figure S3). For each of the bacterial communities, we observed that BA and LA metabolite profiles were typically very similar to each other, but different from the profiles measured after fermentation of HA or FOS.

In order to identify effects of the fermentation substrates on the metabolome and avoid masking caused by the differences between the individual bacterial communities PCA analysis was carried out for each community separately and presented as bi-plots (Figures 2 and 3). Based on this, metabolites causing the differences between samples fermented on the four substrates were chosen (Tables 3 and 4, Figure 4). The above described observation that profiles obtained after BA and LA fermentation were typically similar to each other, but different from HA and FOS fermented samples, was also true in the PCA plots based on the individual bacterial communities. In general, the metabolite contents of FOS fermented samples were more different from all three samples based on arabino-oligosaccharide fermentation, than these three samples were from each other.

Phenylalanine (Figure 2; No. 1; all individuals), xanthine (Figure 3; No. 18; B2, B3, B5 and B6) and linoleic acid or its derivative (Figure 3; No. 21; B1, B4 and B6) contributed significantly to the difference between arabino-oligosaccharide and FOS-fermented samples. In all six microbial communities, phenylalanine (1) was present in higher

amounts in one or more of the AOS-fermented samples than in FOS-fermented samples. Metabolites which were present in high levels in FOS and HA fermentation samples, but separated these from BA and LA fermentations, were N'-acetylspermidine (Figure 2; No. 2; B1, B2, B3, B5 and B6), phenyllactic acid (Figure 3; No. 19; B2, B3, B4, B5 and B6) and a flavonoid (Figure 3; No. 16; B2, B3, B4, B5). A significant increase of N'-acetylspermidine, phenyllactic acid and flavonoid was observed in all subjects after fermentation of one or more of the 4 oligisaccharides (Figure 4), however, in general the highest increase of this metabolite was in HA and FOS. BA and LA fractions seems to stimulate production of cysteine (Figure 2; No. 6; B1, B3, B5 and B6), aminobenzoic acid (Figure 2; No. 8; B1, B4 and B5), hypoxanthine (Figure 2; No. 9; B2, B3, B4 and B6) and 3-oxooctadecanoic acid (Figure 2, No. 11; B3, B5, B6 and Figure 3; No. 17; B2, B3, B5, B6). PCA plots also showed a positive correlation between 3-oxoalanine (Figure 2; No. 4; B1, B2, B3, B5, B6), tyramine (Figure 2; No. 5; B1, B2; B3; B5, B6), homoveratic acid (Figure 3; No. 13 B1, B2; B3; B5, B6); and arabionofuranosyl structures (Figure 3; No. 15; B1, B2, B3, B4 and B5) with the presence of high molecular weight arabino-oligosaccharides (HA). Unspecified metabolite (Figure 2; No. 7; B1, B2, B3, B4, B5), (R)-3-hydroxy-octadecanoic acid (Figure 2, No. 12; B4, B5, B6), fatty acid derivatives (Figure 3, No. 14 and 20; all individuals) and allantoic acid (Figure 3; No. 22; B3, B4, B6) were mostly correlated to FOS, however this could not be confirmed by P-value calculations (Figure 4). Presence of iso-valeraldehyde and oleamide (Figure 2, No. 3 and 10 respectively) depended highly on the bacterial community.

**Combined analysis of bacteria and metabolites**

A PCA analysis was conducted for combined LCMS and PCR data. A loading plot combining selected metabolites (Table 3 and 4) with all targeted bacteria taxa (Table 1) was created (Figure 5) in order to reveal correlations between the presence of specific bacteria and specific metabolites.

## Discussion

Previous *in vitro* studies with sugar beet arabino-oligosaccharides (AOS) have showed their bifidogenic effect and influence on the gastrointestinal microflora [10,11]. Our present results confirmed that AOS, whether it was high molecular weight (HA), low molecular weight (LA), or a mix of these (BA) selectively stimulated the growth of bifidobacterial species (Table1 and 2), which are associated with positive effects on the host health [22-24]. Another potentially positive aspect of the microbiota modulation was a decrease of *Desulfovibrio* spp. (Table 1 and 2), which were seen after both FOS and HA fermentation. This species belongs to the sulfate reducing organisms, which are suggested to play a role in inflammatory bowel diseases (IBD) due to the toxic effects of sulphide on colonic epithelial cells [25-29]. Additionally, an increased abundance of bacterial species within the genus *Allistipes* has been correlated with a greater frequency of pain in patients with IBD [30], while we found that the amount of *Allistipes* spp.was reduced by fermentation of LA and FOS. Most of the measured bacterial taxa were not affected by fermentation of AOS or FOS (Table 2). As also observed for the measured bacterial taxa (Figure S1), the metabolite profile resulting from fermentation of each of the oligosaccharides depended varied between the individual microbial communities (Figures S2 and S3). However, PCA plots based on samples from each of the communities (Figures 2 and 3) revealed a number of specific metabolites, which were typically seen to differ dependent of substrate (oligosaccharide) source (Tables 3 and 4). Typically, we observed that BA and LA metabolite profiles were similar to each other, but different from the profiles measured after fermentation of HA or FOS.

Metabolites which increased after fermentation with AOS included phenylalanine, xanthine, linoleic acid or its derivatives. A possible source of phenylalanine was bacterial degradation of arabionofuranosyl structures, present in the AOS [11]. Arabionofuranosyl structures were partly causing the observed difference between HA and LA or BA, respectively. The HA fermentation fraction, which was enriched for high- mass carbohydrates, was also higher in feruloylated AOS (Table 4). It has previously been suggested that non-digestible carbohydrates with low mass reach the proximal colon, while non-digestible carbohydrates with higher mass travel all the way to the distal colon

[31]. Therefore, ingestion of HA and LA fractions may result in alterations in different parts of the bowel. Additionally, increased amounts of feruloylated AOS in the HA fraction may lead to a higher probability of the non-digestible carbohydrate to reach the distal colon, and prevent the accumulation of toxic by-products of proteolysis and amino acid fermentation, which takes place mainly when carbohydrates are absent [32,33]. Decomposition of the feruloylated AOS by the intestinal microbiota might lead to an increase in the amount of phenylalanine available for epithelial cells. This essential amino acid is a precursor for tyrosine, signaling molecules such as dopamine, noradrenaline and adrenaline as well as skin pigment – melanin [20,34].

A flavonoid released during fermentation was mostly correlated to HA and FOS in the PCA plots (Figure 3). However, a significant increase was observed for all of the tested carbohydrates (Figure 4). Flavonoids may be released by microbial fermentation of plant structures present in the fecal matter. Flavonoids are shown to have various biological effects on the human body. Researchers have a key interest in the antioxidative ramification of those polyphenolic compounds against cancer, atherosclerosis and chronic inflammation [35,36], as they are known to inhibit and induce a large number of mammalian enzymes [37] involved in e.g. cell division, proliferation and detoxycation [38]. Absorption of flavonoids from the diet was long considered to be negligible, as they are present in foods bound to sugars as β-glycosides [39]. It has now been shown that the final biological activity of flavonoids depends on the intestinal bacterial metabolism, which breaks the β-glycosidic bondsand leads to biotransformation of some flavonoic compounds, thereby changing their bioactivity [40]. Many Lactic Acid Bacteria (LAB)are able to brake the β-glycosidic bonds, and the observed increase in the number of *Bifidobacteria* may have caused a higher amount of free flavonoids to be released, as observed in the HA fraction and FOS. Xanthine, hypoxanthine and allantoic acid are all products of purine metabolism [34]. Fermentations with all of the tested oligosaccharides (Figure 4, No. 9, 18, 22) showed a significant increase in the abundance of these metabolites, indicating an activation in this pathway compared to the NC incubations. Linoleic acid belongs to the group of essential unsaturated fatty acids, which humans are not able to synthesize. One of the health promoting properties of bifidobacteria is production of bioactive acids, namely the conjugated linoleic acid (CLA) [41], which is produced from linoleic acid available in the GIT, originating e.g. from consumed plant

tissues. Fermentations with non-digestable carbohydrates is known to cause an increase in CLA production. In this study, a positive correlation between the linoleic acid (No. 21) and bifidobacteria (Bis), was found (Figure 5).

The metabolite production resulting from fermentation of HA was seen to differ from that resulting from fermentation of either LA or BA (Figures 2 and 3). The HA fraction had a greater impact on the production of 3-oxoalanine, tyramine and homoveratic acid. 3-oxoalanine is found as an oxidation product of cysteine or serine containing substrates in anaerobic conditions [42,43]. In relation to the cysteine, mostly present in the fermentations with LA and BA (Figure 2, No. 6), this could possibly be an evidence of different sulfate containing amino acids metabolism in the high-mass fraction, in relation to the pathway or turnover speed. Consumption of tyramine, which is produced mainly by lactic acid bacteria (LAB), is reported to cause allergies, migraine and heart failure in very high concentrations [44,45]. However, this is in relation to the dietary products already high in the concentration of tyramine and not the production of this essential monoamine by intestinal bacteria, in case of which many co-metabolic relations are still highly unstudied. Homoveratic acid is a metabolite found in urine samples [46,47], plant cells [48] and microbial cells [49]. Both, homoveratic acid and tyramine may have an effect on eukaryotic endocrine metabolic pathways. Fermentation of AOS, and particularly the HA fraction, was seen to increase the abundance of these metabolites. However, considering the phenolic structure of homoveratric acid and tyramine, it could also be a product of bacterial degradation of ferulic structures present in the AOS fractions.

The HA fraction also had some part of the metabolic response in common with FOS, as N'-acetylspermidine and phenyllactic acid were increased typically in HA as well as FOS fermentations. N'-acetylspermidine have substantial and fundamental roles in various biological systems including mammals, plants and microbes [20]. Whether bacterial N'-acetylspermidines affect epithelial cell growth and proliferation remains to be addressed. Phenyllactic produced by LAB, has been shown to prevent growth of pathogens [50,51]. Figure 5 suggests that the abundance of phenyllactic acid is rather related to the presence of *Lactobacillus* than to *Bifidobacterium*, however the *Lactobacillus* populations did not increase during fermentations (Figure S1). While the metabolite profiles produced by BA and LA fermentation in all the individual microbiotas were different from those produced by

HA and FOS (Figures 2 and 3), no significant differences in the metabolic profiles were observed between the BA and LA fractions (Figures 2, 3 and 4). We speculate that intestinal bacteria were more prone to metabolize the carbohydrates with lower mass, which were present in rather high amounts in BA (Figure 1). Metabolites typically correlated with the BA and LA fractions were cysteine, aminobenzoic acid and 3-oxooctadecanoic acid. Cysteine may arise from bacterial metabolism of plant structures [34], and it has been shown that free cysteine contributes to the maintenance of anaerobic conditions by binding free oxygen [52]. This may be important for human health by preventing formation of free radicals in the intestines. Cysteine is also known to increase the pH during fermentation by buffering the environment [53]. However, high pH in the intestine is not desired, and low pH is known to have anti-cancerogenic effect in the human colon [54,55]. High amounts of cysteine, may increase the pH in the fermentation cultures, and may explain the higher number of *Desulfovibrio* spp. observed in the BA and LA fermentations (Table 2), since this species may have a competitive advantage at high pH. Additionally, previous reports show that cysteine supports the growth of this *Desulfovibrio* [56]. Aminobenzoic acid could be originating from degradation of phytochemicals or ferulic structures (present in the AOS fractions) by the microbial communities [34]. It has previously been suggested that the intestinal microbiota transforms phenolic compounds into bioactive forms, which are anticipated to have a positive influence on the human health [57]. 3-Oxooctadecanoic acid (11, 17) and (R)-3-hydroxy-octadecanoic acid (12) are building blocks of the unsaturated fatty acids [34]. 3-Oxooctadecanoic acid (17) was seen to increase in all six microbiotas after fermentation of at least two of the AOS fractions, and also increased in four out of six microbiotas after FOS fermentation (Figure 4). Additionally, differences between abundance of fatty acid derivatives no. 14 and 20 suggest that AOS and FOS might have an impact on the unsaturated fatty acid metabolism carried out by the intestinal microbiota, but that different turnover rates and different pathways are used, [34], depending on the microbiota composition as well as on the type of oligosaccharide.

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**Table 1.** Relative fold change of bacteria target from samples incubated with either BA, LA, HA or FOS compared to the NC samples (set to 1).

| | | Substrates | | | |
|---|---|---|---|---|---|
| **ID** | **Bacterial taxa** | **BA** | **LA** | **HA** | **FOS** |
| F | *Firmicutes* phylum | 0.80 (±0.07) | 0.78 (±0.07) | 0.80 (±0.08) | 0.96 (±0.07) |
| Cc | • *C. coccoides* group | 0.59 (±0.11) | 0.54 (±0.16)* | 0.68 (±0.09) | 0.85 (±0.11) |
| Rs | ○ *Roseburia* spp. | 1.18 (±0.09) | 1.18 (±0.10) | 1.07 (±0.12) | 1.16 (±0.17) |
| Cl | • *C. leptum* subgroup | 0.97 (±0.15) | 0.86 (±0.17) | 0.85 (±0.11) | 0.94 (±0.11) |
| Ls | • *Lactobacillus* spp. | 1.17 (±0.12) | 1.10 (±0.13) | 1.11 (±0.09) | 1.14 (±0.11) |
| B | *Bacteroidetes* phylum | 0.90 (±0.13) | 0.90 (±0.10) | 0.80 (±0.13) | 0.80 (±0.09) |
| Bs | • *Bacteroides* spp. | 0.82 (±0.15) | 0.80 (±0.12) | 0.74 (±0.13) | 0.70 (±0.14) |
| Bf | ○ *Bac. fragilis* group | 0.92 (±0.21) | 0.91 (±0.15) | 0.85 (±0.21) | 0.78 (±0.14) |
| Ps | • *Prevotella* spp. | 0.96 (±0.13) | 0.93 (±0.10) | 0.97 (±0.08) | 0.99 (±0.10) |
| As | • *Alistipes* spp. | 0.80 (±0.07) | 0.74 (±0.06)* | 0.77 (±0.06) | 0.71 (±0.04)* |
| | *Actinobacteria* | | | | |
| Bis | • *Bifidobacterium* spp. | 1.72 (±0.28)** | 1.72 (±0.26)*** | 1.79 (±0.24)*** | 1.64 (±0.22)** |
| | *Verrucomicrobia* | | | | |
| Am | • *Akk. Muciniphila* | 1.26 (±0.09) | 1.28 (±0.09) | 0.80 (±0.13) | 0.96 (±0.04) |
| | *Proteobacteria* | | | | |
| E | • *Enterobacteriaceae* | 0.90 (±0.09) | 1.14 (±0.09) | 1.10 (±0.10) | 0.94 (±0.25) |
| Ds | • *Desulfovibrio* spp. | 0.81 (±0.05) | 0.80 (±0.04) | 0.66 (±0.05)** | 0.74 (±0.07)* |

All calculated data are means±SEM of the six fecal communities. Asterisks designate a significant difference from samples taken after 24 hours of incubation without any added carbon-source (NC samples) (P<0.05 (*), P<0.01 (**), P<0.001 (***)). No significant difference in the density of bacterial taxa after fermentation was obtained when comparing the four substrates.

**Table 2.** Relative fold change of bacteria target in samples incubated with BA, LA, HA or FOS compared to the NC samples (set to 1) for each of the six fecal communities.

| | | Substrates | | | |
|---|---|---|---|---|---|
| | Community | BA | LA | HA | FOS |
| *Bifidobacteria* | 1 | 1.32 (±0.03)$^{b}$ | 1.34 (±0.02)$^{b}$ | 1.35 (±0.02)$^{b}$ | *1.01 (±0.02)*$^{a}$ |
| | 2 | 1.71 (±0.05)$^{a}$ | 1.67 (±0.03)$^{a}$ | 1.75 (±0.02)$^{a}$ | 1.63 (±0.10)$^{a}$ |
| | 3 | 1.84 (±0.02)$^{a}$ | 1.91 (±0.04)$^{a}$ | 2.17 (±0.14)$^{ab}$ | 2.46 (±0.04)$^{b}$ |
| | 4 | 1.29 (±0.04)$^{ab}$ | 1.34 (±0.03)$^{b}$ | 1.35 (±0.05)$^{b}$ | *1.16 (±0.03)*$^{a}$ |
| | 5 | 1.25 (±0.02)$^{a}$ | 1.30 (±0.02)$^{a}$ | 1.40 (±0.01)$^{ab}$ | 1.56 (±0.02)$^{b}$ |
| | 6 | 5.54 (±0.06)$^{a}$ | 5.54 (±0.05)$^{a}$ | 5.11 (±0.07)$^{b}$ | 3.85 (±0.07)$^{c}$ |
| *C. coccoides* group | 1 | *0.93 (±0.01)*$^{a}$ | *1.00 (±0.02)*$^{ab}$ | *1.14 (±0.05)*$^{b}$ | *1.09 (±0.06)*$^{ab}$ |
| | 2 | 0.60 (±0.01)$^{b}$ | 0.25 (±0.06)$^{c}$ | 0.64 (±0.01)$^{ab}$ | *0.85 (±0.03)*$^{a}$ |
| | 3 | 0.21 (±0.02)$^{ab}$ | 0.20 (±0.03)$^{b}$ | 0.44 (±0.03)$^{c}$ | 0.36 (±0.03)$^{ac}$ |
| | 4 | *0.66 (±0.03)*$^{a}$ | *0.86 (±0.13)*$^{ab}$ | *0.62 (±0.16)*$^{a}$ | *1.20 (±0.01)*$^{b}$ |
| | 5 | 0.28 (±0.02)$^{a}$ | 0.25 (±0.04)$^{a}$ | 0.33 (±0.01)$^{a}$ | 0.68 (±0.02)$^{b}$ |
| | 6 | 0.46 (±0.01)$^{a}$ | 0.33 (±0.09)$^{a}$ | 0.49 (±0.04)$^{a}$ | *0.98 (±0.04)*$^{b}$ |
| *Alistipes* spp. | 1 | *0.97 (±0.02)*$^{a}$ | 0.91 (±0.02)$^{a}$ | 0.77 (±0.03)$^{b}$ | 0.77 (±0.01)$^{b}$ |
| | 2 | *0.79 (±0.05)*$^{a}$ | 0.60 (±0.06)$^{a}$ | *0.80 (±0.08)*$^{a}$ | 0.67 (0.07)$^{a}$ |
| | 3 | 0.73 (±0.01)$^{a}$ | 0.72 (±0.01)$^{a}$ | *0.82 (±0.06)*$^{a}$ | *0.79 (±0.07)*$^{a}$ |
| | 4 | 0.70 (±0.01)$^{a,b}$ | 0.76 (±0.04)$^{a}$ | 0.62 (±0.03)$^{b}$ | 0.78 (±0.01)$^{a}$ |
| | 5 | 0.58 (±0.02)$^{a}$ | 0.55 (±0.05)$^{a}$ | 0.59 (±0.03)$^{a}$ | 0.51 (±0.01)$^{a}$ |
| | 6 | *1.01 (±0.02)*$^{a}$ | *0.86 (±0.06)*$^{b}$ | *0.99 (±0.03)*$^{a,b}$ | 0.71 (±0.02)$^{c}$ |
| *Desulfovibrio* spp. | 1 | *0.95 (±0.04)*$^{a}$ | *0.81 (±0.03)*$^{a}$ | 0.50 (±0.07)$^{a}$ | *0.78 (±0.19)*$^{a}$ |
| | 2 | *0.90 (±0.02)*$^{a}$ | *0.88 (±0.02)*$^{a}$ | 0.55 (±0.03)$^{b}$ | 0.69 (±0.01)$^{b}$ |
| | 3 | 0.76 (±0.01)$^{a,c}$ | *0.87 (±0.02)*$^{a}$ | 0.50 (±0.05)$^{b}$ | 0.62 (±0.09)$^{b,c}$ |
| | 4 | *0.79 (±0.01)*$^{a}$ | *0.82 (±0.02)*$^{a}$ | 0.68 (±0.04)$^{a}$ | 0.70 (±0.02)$^{a}$ |
| | 5 | 0.67 (±0.02)$^{a}$ | 0.64 (±0.04)$^{a}$ | 0.67 (±0.04)$^{a}$ | 0.56 (±0.08)$^{a}$ |
| | 6 | *0.84 (±0.01)*$^{a}$ | 0.69 (±0.13)$^{a}$ | *0.78 (±0.08)*$^{a}$ | *0.94 (±0.06)*$^{a}$ |

All calculated data are means ± SEM of triplicate fermentations. Results in the same row followed by different roman letters a-c indicate significant difference in bacterial abundance between the four substrates. Italic indicates no significant difference from NC sample (P>0.05).

**Table 3.** Annotated metabolites from LCMS analysis in positive mode found to separate fermentation samples according to carbohydrate source (Figure 2).

| Number | RT [min] | $M_{LCMS}$ [Da] | Adduct | $M_{REF}$ [Da] | Error [mDa] | $M_{MSMS}$ | Metabolite candidate |
|---|---|---|---|---|---|---|---|
| **1** | 1.7 | 166.086493 | M+H | 166.086255 | 0.2 | 120.081723 | phenylalanine |
| **2** | 0.8 | 188.175739 | M+H | 188.175738 | 0.0 | 171.150205<br>128.071353<br>117.103928<br>100.077105<br>64.024444 | N'-Acetylspermidine |
| **3** | 0.8 | 104.106928 | M+$NH_4$ | 104.106988 | 0.1 | NF | Iso-Valeraldehyde |
| **4** | 1.5 | 121.062386 | M+ $NH_4$ | 121.060766 | 1.6 | 103.054712<br>93.069976<br>57.993688 | 3-Oxoalanine |
| **5** | 1.1 | 138.091331 | M+H | 138.091340 | 0.0 | 121.064816<br>93.070169 | Tyramine |
| **6** | 0.9 | 139.050163 | M+$NH_4$ | 139.0502 | 0.0 | 121.039480 | Cysteine |
| **7** | 0.7 | 140.068238 | M+Na | 140.068197 | 0.1 | 96.078471 | Betaine<br>5-Aminopentanoic acid<br>N-Methyl-a-aminoisobutyric acid<br>Valine<br>Norvaline |
| | 0.7 | 156.042329 | M+K | 156.042137 | 0.2 | NF | |
| | 0.7 | 257.147484 | 2M+Na | 257.147175 | 0.3 | 140.069251 | |
| **8** | 1.3 | 155.081504 | M+$NH_4$ | 155.0815 | 0.0 | NF | Aminobenzoic acid |
| **9** | 1.0 | 137.045787 | M+H | 137.045787 | 0.0 | NF | Hypoxanthine |
| **10** | 6.6 | 282.279062 | M+H | 282.279141 | 0.0 | 265.253039<br>247.245066<br>149.024021<br>142.122957<br>114.091440<br>111.117935<br>70.272614 | Oleamide |
| **11** | 6.3 | 321.240053 | M+Na | 321.240013 | 0.0 | NF | 3-Oxooctadecanoic acid |
| **12** | 6.1 | 323.255570 | M+Na | 323.255663 | 0.1 | NF | (R)-3-Hydroxy-octadecanoic acid |

RT – retention time on the chromatogram; $M_{LCMS}$ – *m/z* (mass to charge) ratio measured; $M_{REF}$ – *m/z* ratio ofmetabolites given in the referral data bases; Error – difference between $M_{LCMS}$ and $M_{REF}$; $M_{MSMS}$ – m/z ratio of the fragment ions after MSMS; NF – no fragmentation or fragments not visible in the given MS settings.

**Table 4.** Annotated metabolites from LCMS analysis in negative mode found to separate fermentation samples according to carbohydrate source (Figure 3).

| Number | RT [min] | $M_{LCMS}$ [Da] | Adduct | $M_{REF}$ [Da] | Error [mDa] | $M_{MSMS}$ | Metabolite candidate |
|---|---|---|---|---|---|---|---|
| **13** | 2.9 | 195.066074 | M-H | 195.066283 | 0.2 | 151.094347<br>136.065370<br>121.041572 | Homoveratric acid |
| **14** | 0.8 | 133.070545 | | | | NF | Fatty acid derivative |
| **15** | 3.3 | 457.135071 | | | | 397.114107<br>367.102362<br>325.094322<br>265.075597<br>235.062259<br>193.052703 | Arabionofuranosyl structure |
| **16** | 3.2 | 263.073298 | M-H | 263.0714 | 1.9 | 191.074171<br>165.073565 | Flavonoid |
| **17** | 6.3 | 297.243324 | M-H | 297.243530 | 0.2 | NF | 3-Oxooctadecanoic acid |
| **18** | 1.0 | 151.026420 | M-H | 151.026149 | 0.2 | 108.020397 | Xanthine |
| **19** | 3.2 | 165.055711 | M-H | 165.055718 | 0.0 | 147.048091<br>119.052135<br>103.059652 | Phenyllactic acid |
| **20** | 0.6 | 131.071452 | M-H | 131.071368 | 0.0 | NF | Fatty acid derivative |
| **21** | 6.6 | 279.233124 | M-H | 279.232954 | 0.1 | NF | Linoleic acid<br>Derivative of linoleic acid |
| **22** | 0.9 | 175.049132 | M-H | 175.047272 | 1.9 | 157.036324<br>131.052535<br>113.039588 | Allantoic acid |

RT – retention time on the chromatogram; $M_{LCMS}$ – m/z (mass to charge) ratio measured; $M_{REF}$ – m/z ratio of metabolites given in the referral data bases; Error – difference between $M_{LCMS}$ and $M_{REF}$; $M_{MSMS}$ – m/z ratio of the fragment ions after MSMS; NF – no fragmentation or fragments not visible in the given MS settings.

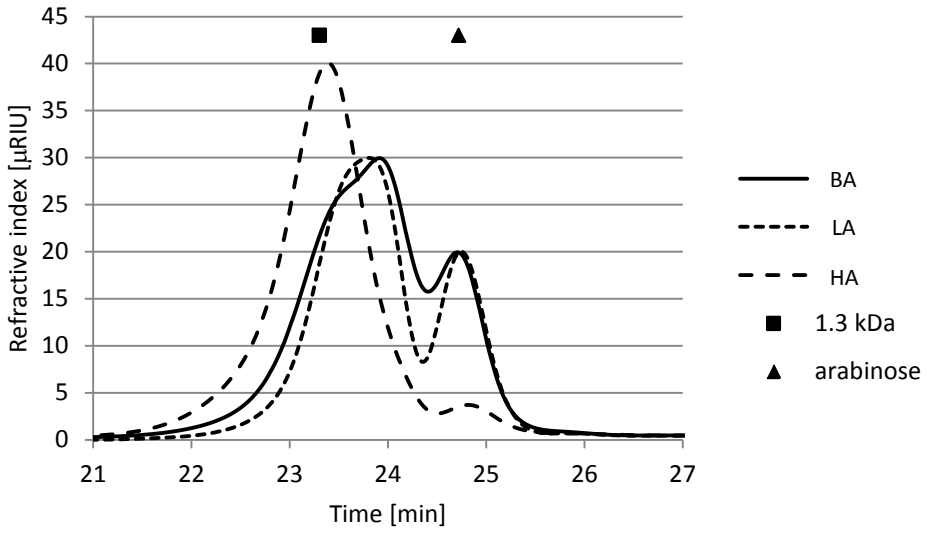



**Figure 1** Size exclusion chromatography of mixed arabino-oligosaccharides (MA), low mass arabino-oligosaccharides (LA), and high mass arabino-oligosaccharides (HA) in comparison with arabinose and 1.3 kDa pullanan standard.

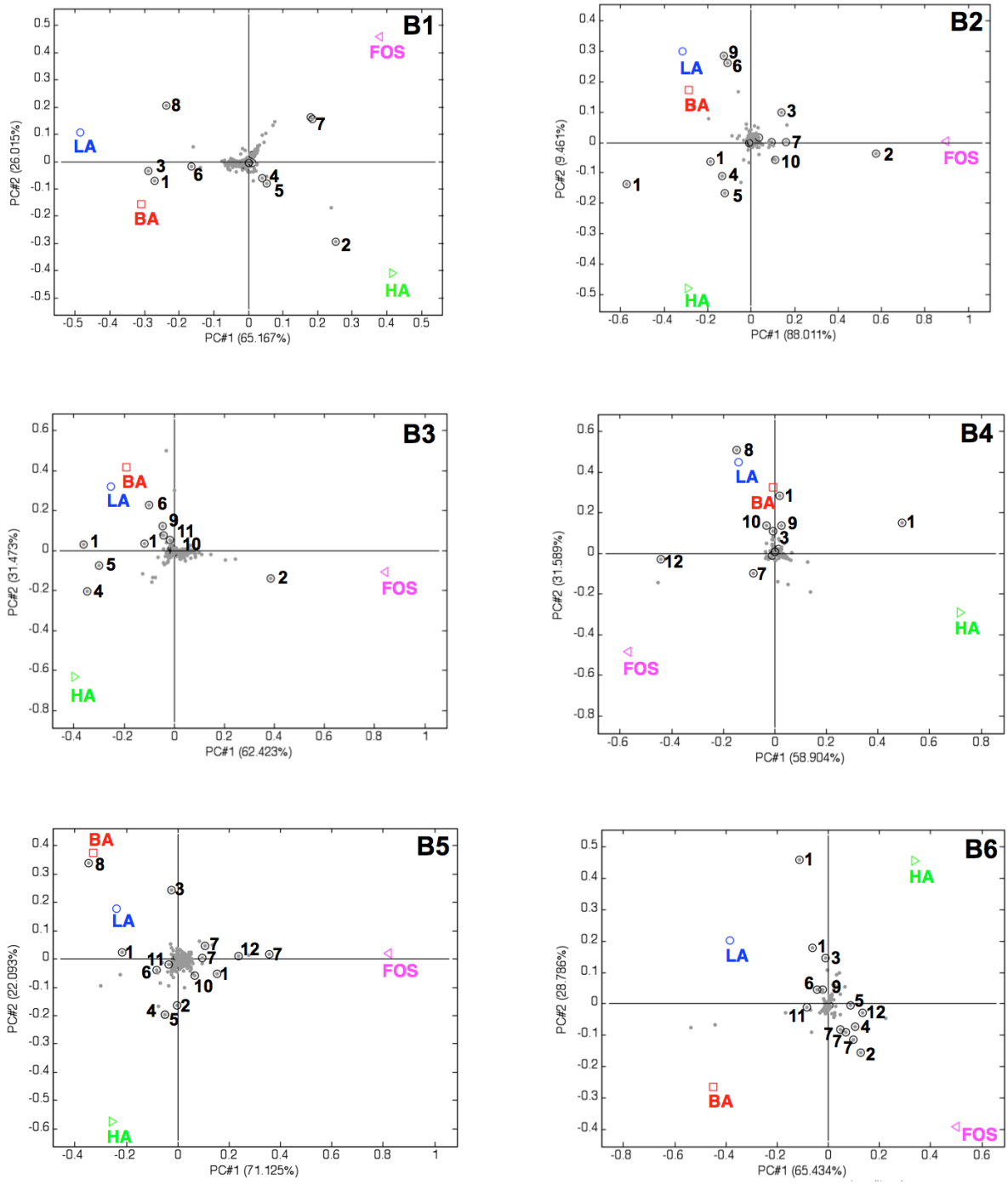



**Figure 2** PCA bi-plots (scores and loadings present on the same plot) of the fermentation samples with different arabino-oligosaccharides BA, LA and HA; and well known prebiotic FOS. Data from LCMS analyses in positive mode for tested bacterial floras separately (B1 – B6). Phenylalanine (1) was observed on the border of the 1 min and 2 min bucket, which is why PCA plots are showing metabolite no. 1 twice. LCMS chromatogram studies (data not shown) confirmed that it was indeed the same metabolite.

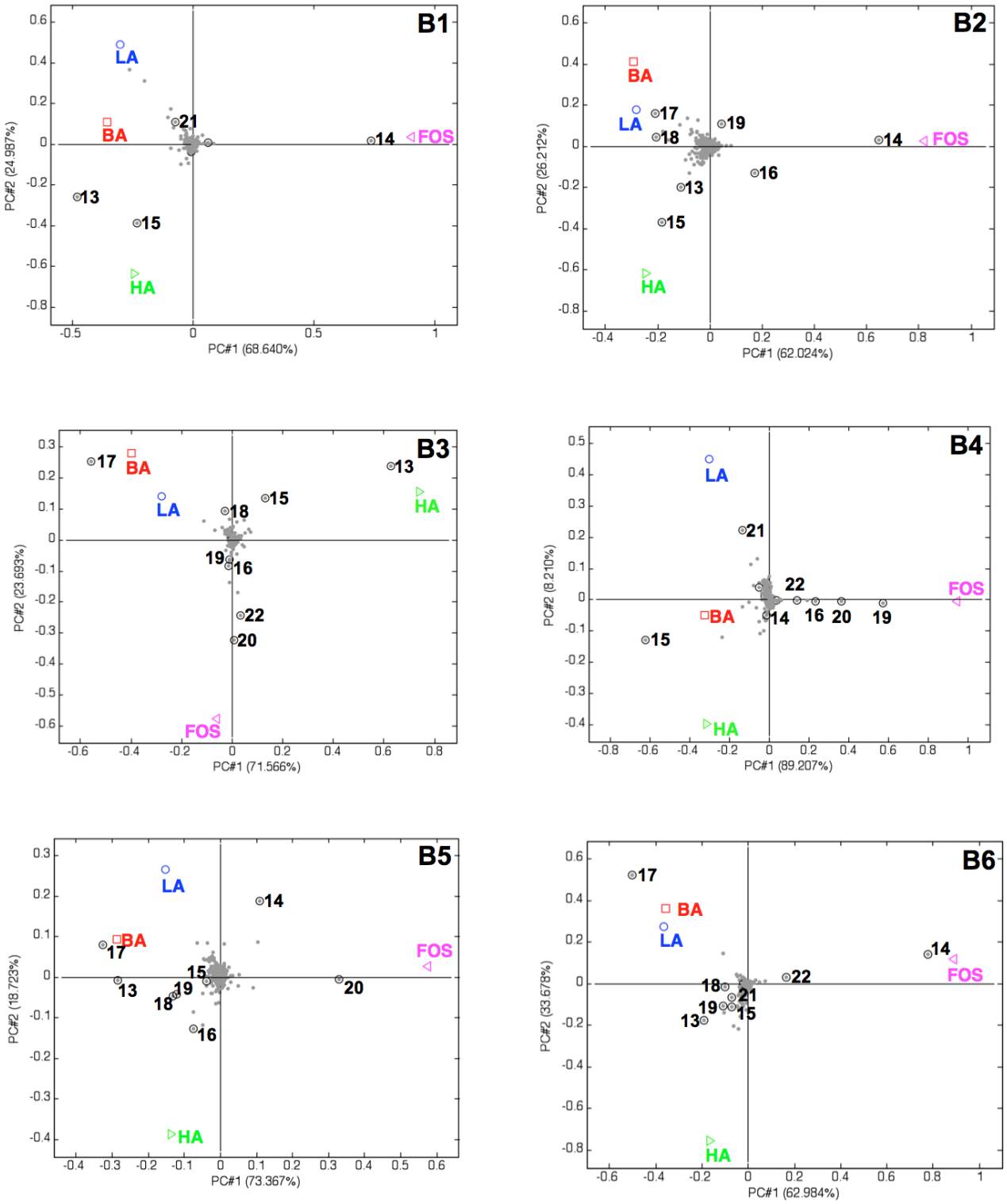



**Figure 3** PCA bi-plots (scores and loadings present on the same plot) of the fermentation samples with different arabino-oligosaccharides BA, LA and HA; and well known prebiotic FOS. Data from LCMS analyses in negative mode for tested bacterial floras separately (B1 – B6).

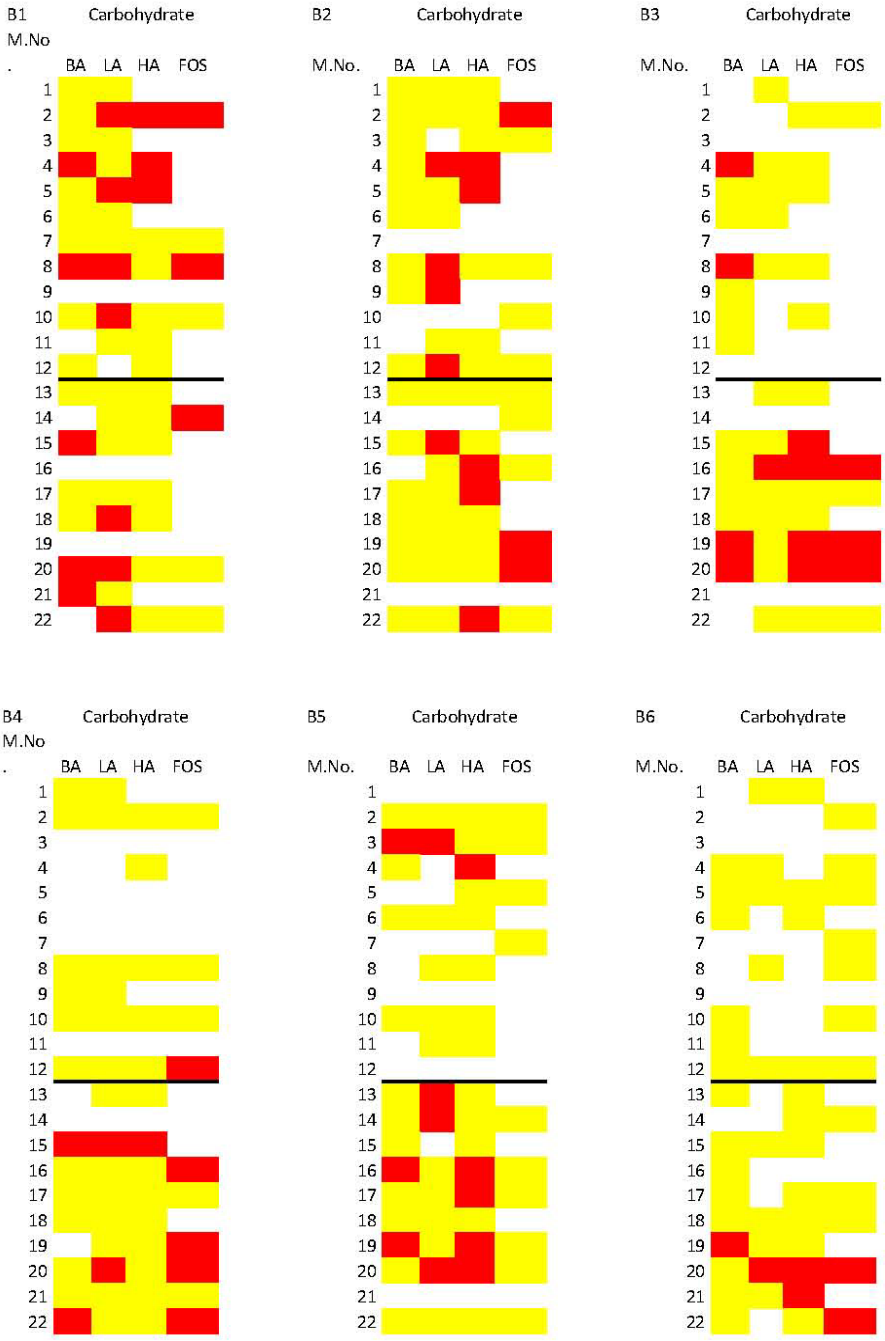



**Figure 4** Heat maps based on the LCMS data of selected metabolites from fermentation samples with BA, LA and HA, and FOS Fermentations in bacterial communities derived from six different individuals (B1-B6) are presented separately (B1-B6). M.No. designates metabolite numbers given in Tables 4 and 5. Red color indicates P-value < 0.005 and yellow color 0.005 < P-value < 0.05 for differences between metabolite abundance before and after fermentation. Only metabolites that increased during fermentation are included. White color indicates a P-value > 0.05 or a change ratio ≤ 1. Metabolites above the black line were found in positive mode, while those below the line were found in negative mode.

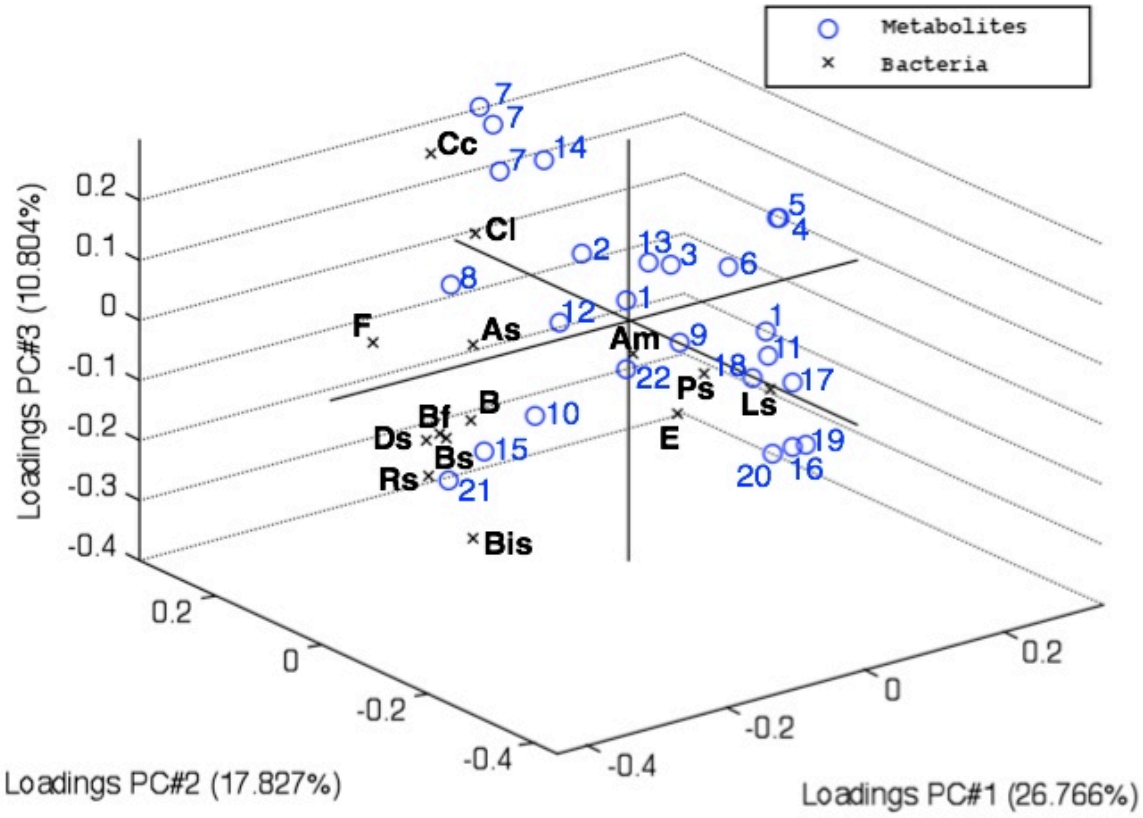


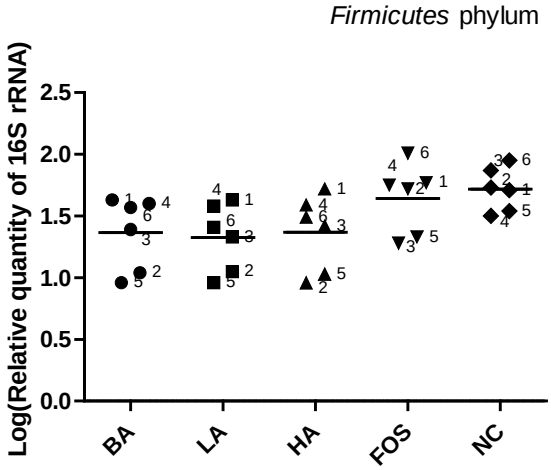

**Figure 5** PCA loading plot of selected metabolites (numbers corresponding to Tabels 3 and 4) combined with microbial abundance data (abbreviations corresponding to Table1).

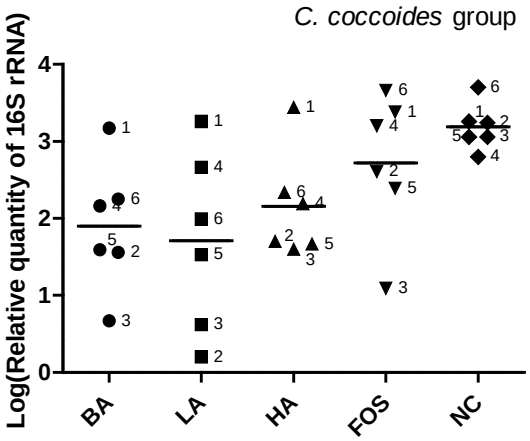
## Supplementary tables and figures

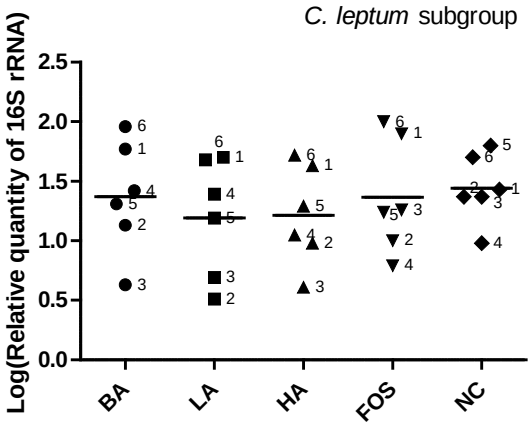
**Table S1** 16S rRNA gene sequences of bacterial taxonomic units.

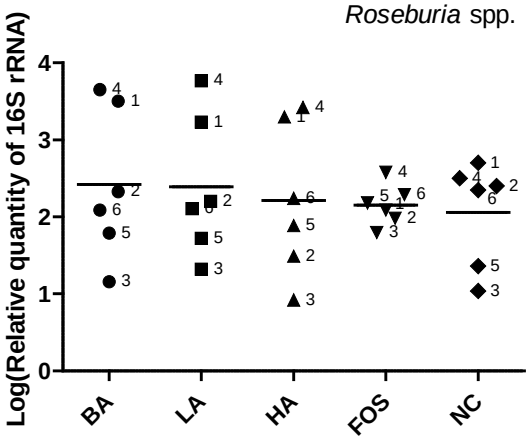
| Target taxon | Primer | No. | Sequence (5'-3') | Fragment size (bp) | Reference |
|---|---|---|---|---|---|
| *Firmicutes* phylum[#] | Firm934F | 1 | gga gya tgt ggt tta att cga agc a | 126 | [58] |
| | Firm1060R | | agc tga cga caa cca tgc ac | | |
| *Clostridium coccoides* group | g-Ccoc-F | 2 | aaa tga cgg tac ctg act aa | 440 | [59] |
| | g-Ccoc-R | | ctt tga gtt tca ttc ttg cga a | | |
| *Clostridium leptum* subgroup | Clep866mF | 3 | tta aca caa taa gtw atc cac ctg g | 314 | [60] |
| | Clept1240mR | | acc ttc ctc cgt ttt gtc aac | | |
| *Roseburia* spp. | RosF | 4 | tac tgc att gga aac tgt cg | 230 | [61] |
| | RosR | | cgg cac cga aga gca at | | |
| *Lactobacillus* spp. | Lacto-F | 5 | agc agt agg gaa tct tcc a | 341 | [62,63] |
| | Lacto-R | | cac cgc tac aca tgg ag | | |
| *Bacteroidetes* phylum | Bact934F | 6 | gga rca tgt ggt tta att cga tga t | 126 | [58] |
| | Bact1060R | | agc tga cga caa cca tgc ag | | |
| *Bacteroides* spp. | BacF | 7 | cga tgg ata ggg gtt ctg aga gga | 238 | Unpublished |
| | BacR | | gct ggc acg gag tta gcc ga | | |
| *Bacteroides fragilis* group | Bfr-F | 8 | ctg aac cag cca agt agc g | 230 | [64] |
| | Bfr-R | | ccg caa act ttc aca act gac tta | | |
| *Prevotella spp.* | PrevF | 9 | cac caa ggc gac gat ca | 283 | [61] |
| | PrevR | | gga taa cgc cyg gac ct | | |
| *Alistipes* spp. | Alis F1-124 | 10 | tta gag atg ggc atg cgt tgt | 320 | [65] |
| | Alis R1-423 | | tga atc ctc cgt att acc gcg | | |
| *Bifidobacterium* spp. | F-bifido | 11 | cgc gtc ygg tgt gaa ag | 244 | [66] |
| | R-bifido | | ccc cac atc cag cat cca | | |
| *Akkermansia muciniphila* | AM1 | 12 | cag cac gtg aag gtg ggg ac | 327 | [67] |
| | AM2 | | cct tgc ggt tgg ctt cag at | | |
| *Enterobacteriaceae* | Eco1457F | 13 | cat tga cgt tac ccg cag aag aag c | 195 | [68] |
| | Eco1652R | | ctc tac gag act caa gct tg | | |
| *Desulfovibrio* spp. | DSV691-F | 14 | ccg tag ata tct gga gga aca tca g | 136 | [69] |
| | DSV826-R | | aca tct agc atc cat cgt tta cag c | | |
| V2-V3 16S rRNA region* | HDA1 | 15 | act cct acg gga ggc agc agt | 200 | [63] |
| | HDA2 | | gta tta ccg cgg ctg ctg gca c | | |

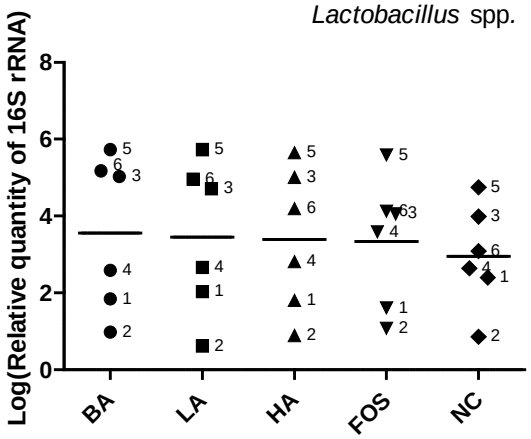
*The HDA primer was used as total bacteria DNA targets in order to normalize, hence correcting differences in total DNA concentration between individual samples.#The primer targets the 16S rRNA gene sequence of the *Firmicutes* phylum and the *Bifidobacterium* group.

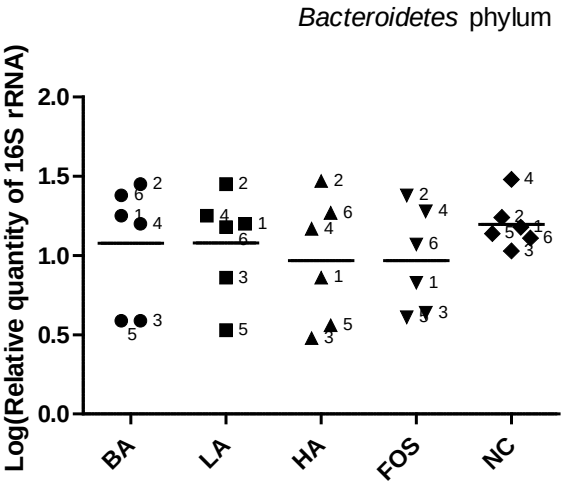

*Firmicutes* phylum
Log(Relative quantity of 16S rRNA)
2.5
2.0
1.5
1.0
0.5
0.0
BA
LA
HA
FOS
NC

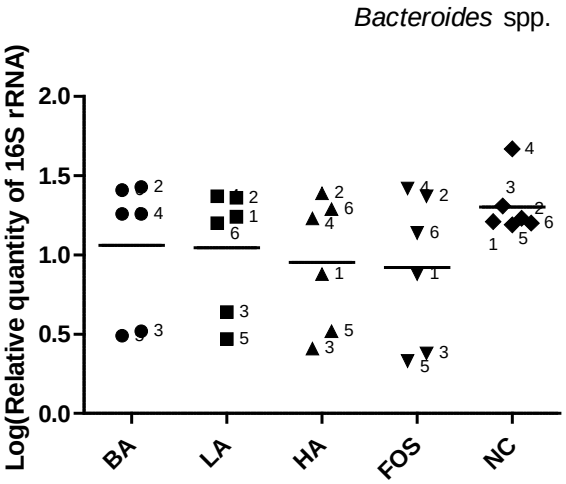

*C. coccoides* group
Log(Relative quantity of 16S rRNA)
4
3
2
1
0
BA
LA
HA
FOS
NC

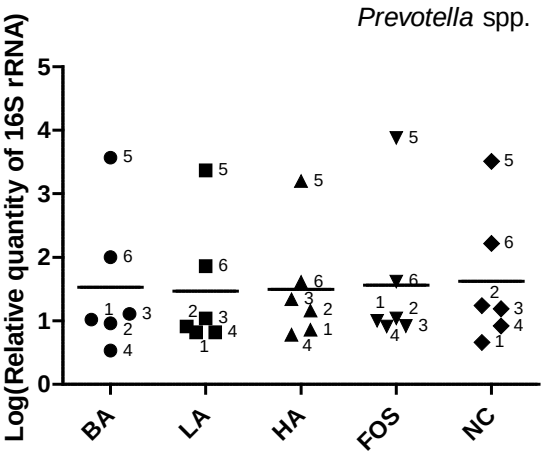

*C. leptum* subgroup
Log(Relative quantity of 16S rRNA)
2.5
2.0
1.5
1.0
0.5
0.0
BA
LA
HA
FOS
NC

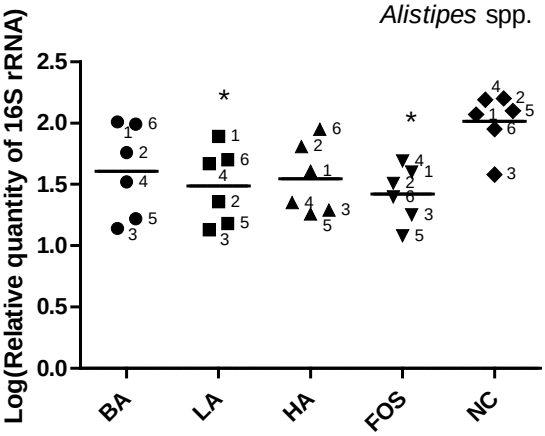

*Roseburia* spp.
Log(Relative quantity of 16S rRNA)
4
3
2
1
0
BA
LA
HA
FOS
NC

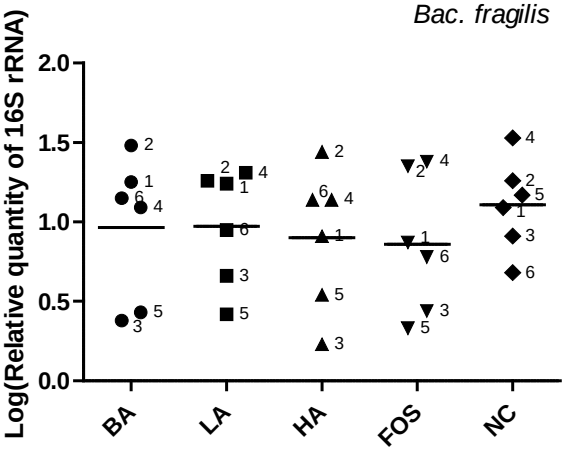

*Lactobacillus* spp.
Log(Relative quantity of 16S rRNA)
8
6
4
2
0
BA
LA
HA
FOS
NC


*Bacteroidetes* phylum
Log(Relative quantity of 16S rRNA)
2.0
1.5
1.0
0.5
0.0
BA
LA
HA
FOS
NC


*Bacteroides* spp.
Log(Relative quantity of 16S rRNA)
2.0
1.5
1.0
0.5
0.0
BA
LA
HA
FOS
NC


*Prevotella* spp.
Log(Relative quantity of 16S rRNA)
5
4
3
2
1
0
BA
LA
HA
FOS
NC


*Alistipes* spp.
Log(Relative quantity of 16S rRNA)
2.5
2.0
1.5
1.0
0.5
0.0
*
*
BA
LA
HA
FOS
NC


*Bac. fragilis*
Log(Relative quantity of 16S rRNA)
2.0
1.5
1.0
0.5
0.0
BA
LA
HA
FOS
NC

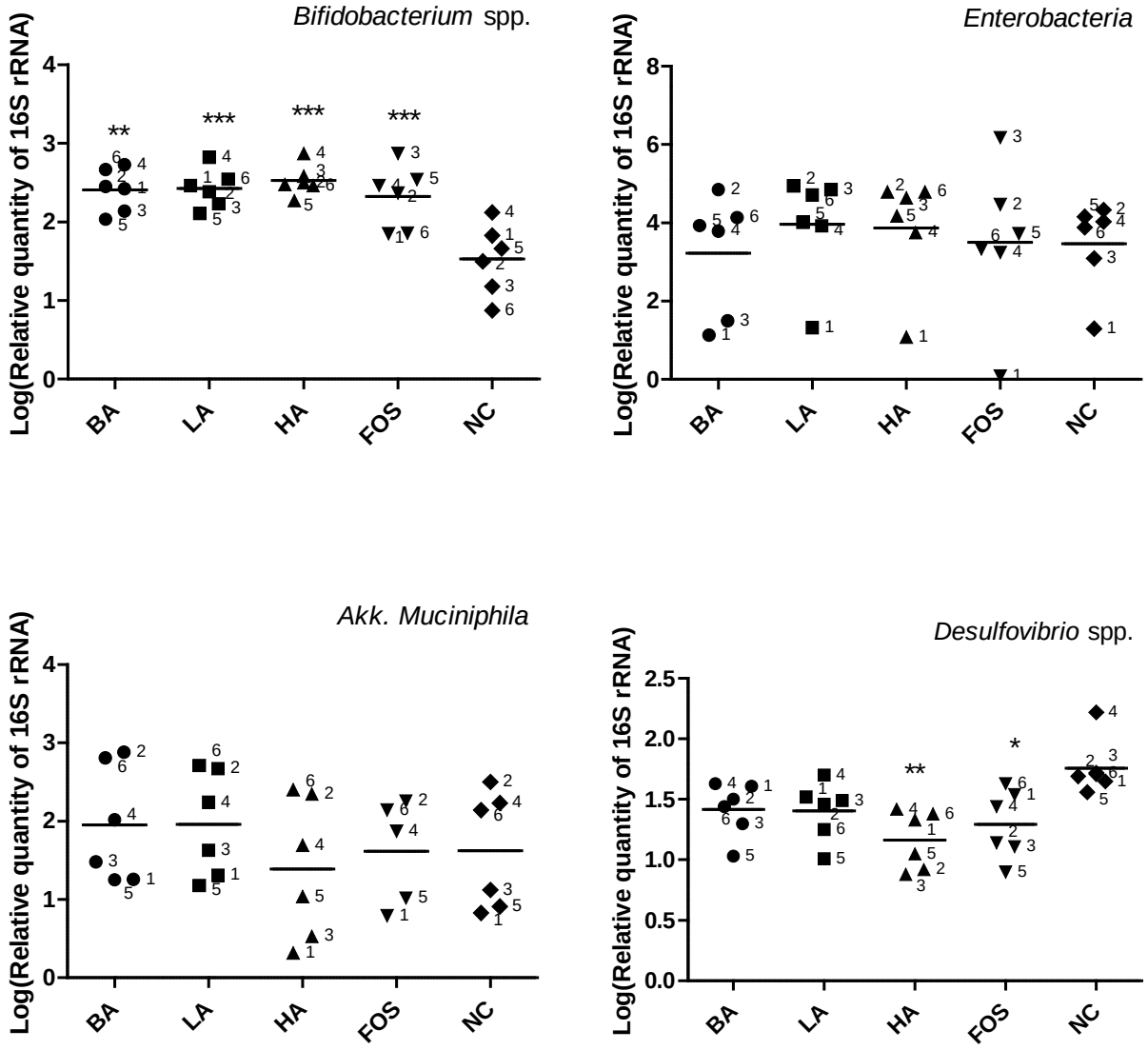



**Figure S1 (3 pages)** Relative quantity of the bacterial taxa in samples incubated with either BA, LA, HA, FOS or no carbon-source added (NC samples) for each of the six fecal communities. The horizontal lines show the mean of the six observations. Asterisks indicate significant differences from the NC samples (P<0.05 (*), P<0.01 (**), P<0.001 (***)).

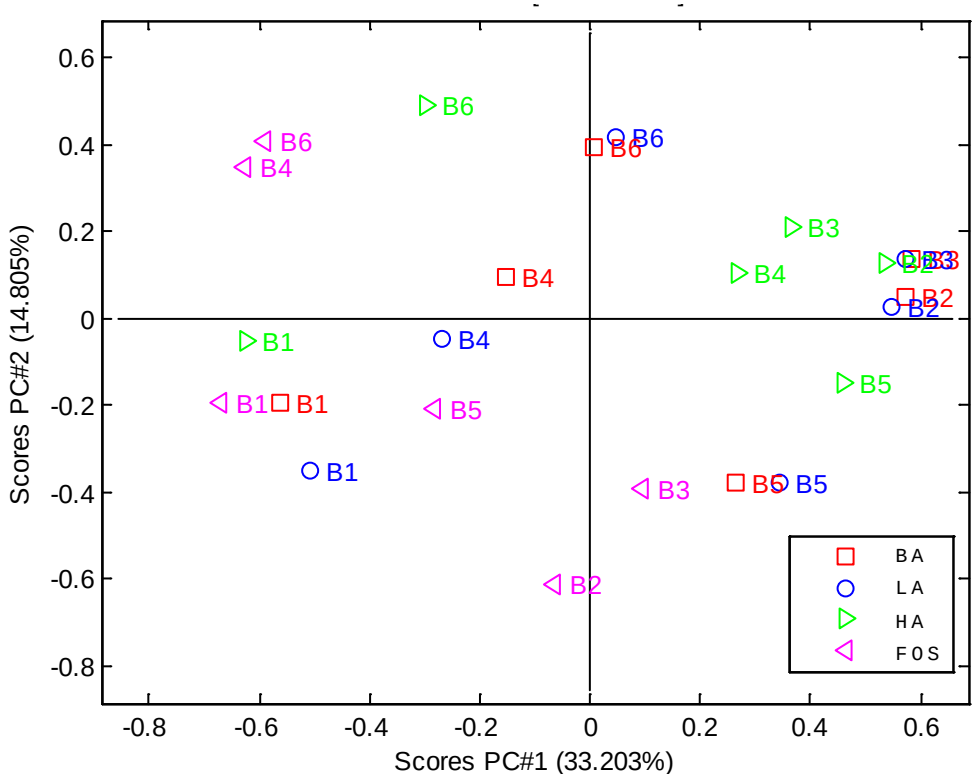



**Figure S2** PCA score plot of the fermentation samples with the different arabino-oligosaccharides BA, LA and HA; and the established prebiotic FOS. Data originate from LCMS analysis in positive mode for all tested bacterial floras (B1 – B6).

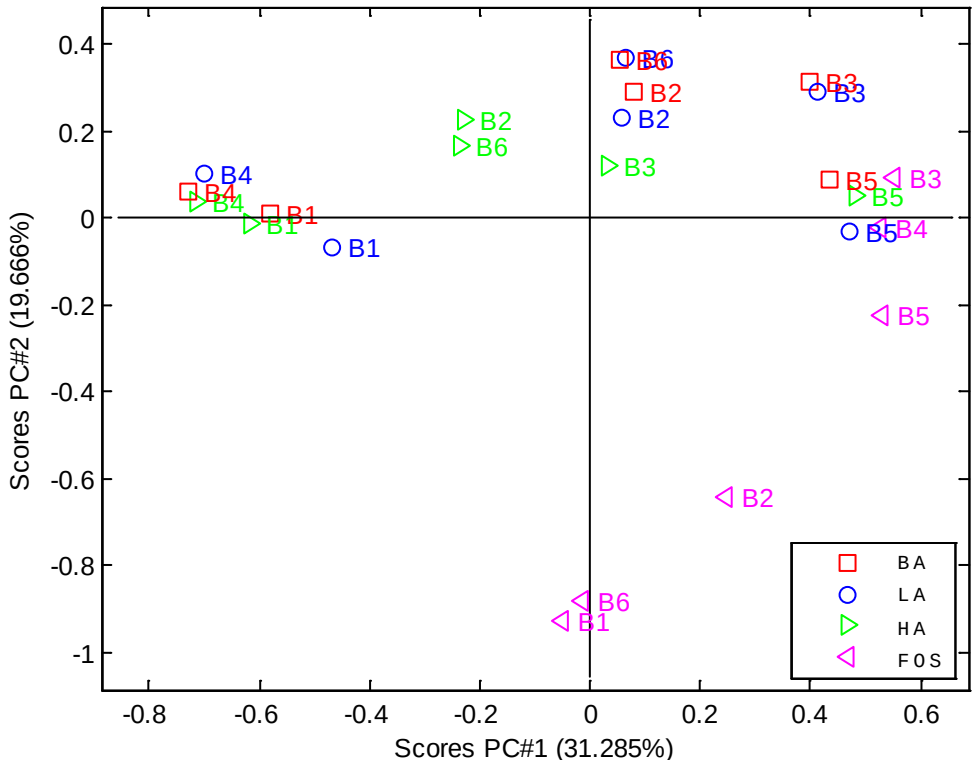



**Figure S3** PCA score plot of the fermentation samples with the different arabino-oligosaccharides BA, LA and HA and the established prebiotic FOS. Data originate from LCMS analysis in negative mode for all tested bacterial floras (B1 – B6).

# Manuscript II

## Microbiotas from UC patients have an altered metabolism and a reduced ability of lactic acid bacteria to colonize mucus

(previously: Fecal bacterial communities derived from Ulcerative Colitis patients display an altered metabolomic profile and a reduced ability of lactic acid bacteria to colonize intestinal mucus in a dynamic in vitro gut model)

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# Microbiotas from UC patients have an altered metabolism and a reduced ability of lactic acid bacteria to colonize mucus

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Running title: Microbial metabolism in Ulcerative Colitis

Key words: Ulcerative Colitis/mucus colonization/lactic acid bacteria/LCMS/ metabolomics/M-SHIME.

## Abstract

We compared fecal microbial communities derived either from Ulcerative Colitis (UC) patients in remission (n=4) or in relapse (n=4), or from healthy subjects (n=4). These communities were used for inoculation of a dynamic *in vitro* gut model (M-SHIME), adapted from the validated Simulator of the Human Intestinal Microbial Ecosystem (SHIME) by incorporation of mucin-covered microcosms. Denaturing Gradient Gel Electrophoresis (DGGE) and quantitative PCR (qPCR) were applied for analysis of the 'luminal' and 'mucosal' microbiota after 42 hours colonization in the model. Liquid Chromatography coupled with Mass Spectrometry (LCMS) was used for analysis of metabolites in the luminal and mucosal samples.

Dice-based cluster analysis of PCR-DGGE fingerprints as well as Principal Component Analysis (PCA) of qPCR data revealed that the microbiota of the 'mucus' largely differed from that of the 'lumen'. This was due to decreased mucus-associated populations of lactic acid producing bacterial populations (LAB) and increased mucus-associated populations of *Roseburia* spp. Importantly, qPCR revealed that LAB originating from UC patients had a significantly decreased capacity to colonize the mucin-covered microcosms as compared to those originating from healthy subjects.

LCMS data indicated that bacterial communities derived from healthy subjects and UC patients in relapse differed with respect to metabolism of phenylalanine, tryptophan, and secondary bile acids. We found significant differences between the metabolomes of UC patients in relapse and remission, respectively, while the metabolome of patients in remission resembled that of healthy subjects.

These novel findings constitute an important contribution to the understanding of the complex etiology of UC.

## Introduction

The mucus layer lining the epithelium of the gastrointestinal tract is important for the protection of the intestinal epithelium in humans. Commensal bacteria have been found to colonize the colonic mucus layer, and previous studies have shown that the microbial community found in the colonic mucus differs from that of the luminal community (Eckburg et al, 2005; Zoetendal et al, 2002). Several microbial characteristics have contributed to the evolvement of the specifically selected mucosal community, including the ability of the bacteria to utilize mucin glycans as energy source as well as resistance to nonspecific antimicrobial peptides and specific antimicrobial immunoglobulins produced by the host (Johansson et al, 2008; Killer &.Marounek, 2011). Additionally, many adhesion molecules expressed by colonic bacteria have mucin glycans as specific epitopes (Kline et al, 2009; Pretzer et al, 2005), and it has been suggested that the glycosylation pattern in mucin, hence the attachment site and energy source for the colonic bacteria, is an important factor for host selection of a specific mucosal community (Johansson et al, 2008). Lack or defects in the mucosal barrier may allow bacteria to reach the epithelium and trigger colonic inflammation.

Ulcerative colitis (UC) is an idiopathic inflammatory bowel disease characterized by chronic relapsing inflammation of the colonic mucosa (Ardizzone, 2003; Kornbluth &.Sachar, 2010). The etiology of UC remains an enigma, and no known infectious agent has been demonstrated (Loftus, 2004; Sartor, 2006). It has been speculated that UC originates from a dysregulated immune response to the commensal intestinal microbiota in genetically susceptible individuals (Brown &.Mayer, 2007; Hanauer, 2006). Human studies have revealed that UC patients have a colonic mucus layer that has an altered O-glycan profile and is significantly thinner than that of healthy subjects, which may select for a different mucosal microbial profile (Larsson et al, 2011; Pullan et al, 1994). Consistently, several studies have shown that patients with UC have an altered bacterial microbiota (Frank et al, 2007;Qin et al, 2010;Sokol et al, 2009;Takaishi et al, 2008). Thus, the bacterial and/or host-bacterial interactions may play a role in the pathogenesis of UC.

*In vitro* models are well-suited to screen the adhering potency of intestinal microbes. They include adhesion assays to various components of the intestinal surface: e.g. intestinal mucus (Ouwehand et al, 2002b), mucins (Van den Abbeele et al, 2009), colonic tissue

(Ouwehand et al, 2002a) or cell lines (Laparra &.Sanz, 2009). A drawback to such models is that they often provide only short-term information based on axenic cultures and thus ignore the interactions between and within the luminal and mucosal microbial communities. Therefore, a dynamic *in vitro* gut model has been developed, which simulates both the luminal and mucosal environment (Van den Abbeele et al, 2011a). This model, named the M-SHIME was adapted from the validated Simulator of the Human Intestinal Microbial Ecosystem (SHIME) (Van den Abbeele et al, 2010). Compared to earlier *in vitro* models, the M-SHIME allows a more representative colonization of specific *Lactobacillus* sp (Van den Abbeele et al, 2011a). Furthermore, high-resolution phylogenetic microbiota profiling shows that the simulated mucosal microbiota is, in correspondence with *in vivo* studies, enriched with *Firmicutes* belonging to the Clostridial clusters IV and XIVa (Van den Abbeele et al, 2010). Moreover, the *in vitro* mucosal environment is necessary to avoid the wash-out of specific surface-associated microbes, which occurs in conventional *in vitro* models. Hence, the M-SHIME allows studies of the mucosal microbiota and the interaction between luminal and mucosal microbial communities.

The aims of this study were (1) to investigate the ability of fecal microbiota from healthy subjects and UC patients in either remission or relapse to colonize the artificial mucus layer of the M-SHIME and (2) to elucidate microbial activity by comparison of metabolic profiles of the luminal and mucosal microbial communities derived from UC patients and healthy subjects. Denaturing Gradient Gel Electrophoresis (DGGE) and quantitative Real-Time PCR (qPCR), both of which are culture-independent methods, were applied for microbiota analysis, while LCMS was used to analyze the potential role of extracellular metabolites produced by microorganisms in the lumen and mucus of the M-SHIME.

## Materials and Methods

### Human volunteers and clinical characteristics of UC patients

Fecal samples were obtained from 8 patients with UC and 4 healthy controls (Langholz et al, 1994). Within the UC group, 4 patients were in clinical remission and 4 patients had active disease at the time of sampling according to clinical and endoscopical criteria

(Binder, 1970). The study was performed in accordance with the Second Helsinki Declaration, reported to the Danish Data Protection Agency and approved by the Regional Ethics Committee. Written informed consent was obtained from each participant under a protocol approved by the Danish National Committee on Biomedical Research Ethics. All four patients with active UC were treated with mesalazine (5-aminosalicylic acid). Two patients with inactive UC received mesalazine, one patient received olsalazine (6-hydroxybenzoate)-salicylic acid), and one received no immunosupressive treatment. None of the participants had been treated with antibiotics for at least 2 months before enrolment and there was no significant difference (P = 0.32) in the mean age of the participants when comparing the 3 groups.

**Sample collection and processing**

Stool samples were collected in airtight containers and stored at 4°C (limited storage time was encouraged (Ott et al, 2004)) until delivery to the laboratory. Feces were homogenized in glycerol to give a 25% feces/glycerol slurry. This was performed in an anaerobic cabinet (Macs Work Station, Don Whitley, containing 10% H2, 10% CO2, and 80% N2). The processed samples were stored at -80°C until further analysis.

**Growth medium and chemicals**

Unless stated otherwise, chemicals were obtained from Sigma (Bornem, Belgium). The M-SHIME feed contained 1.0 g/l arabinogalactan, 2.0 g/l pectin, 1.0 g/l xylan, 3.0 g/l starch, 0.4 g/l glucose, 3.0 g/l yeast extract, 1.0 g/l peptone, 4.0 g/l mucin, and 0.5 g/l cystein. Pancreatic juice contained 12.5 g/l NaHCO3, 6.0 g/l bile salts (Difco, Bierbeek, Belgium) and 0.9 g/l pancreatin. Mucin agar was prepared by boiling autoclaved distilled $H_2O$ containing 5% porcine mucin type II and 1% agar. The pH was adjusted to 6.8 with 10 M NaOH.

**M-SHIME**

Analysis in the M-SHIME dynamic gut model was carried out as previously described (Vermeiren et al, 2012) at two different occasions with six participants for each 42-hours run (two healthy, two UC patients in remission and two UC patients in relapse). The position (vessel) of the inocula from either healthy subjects or UC patients was changed for each run.

**Microbial activity in terms of short-chain fatty acids (SCFA)**

Acetate, propionate, butyrate, valerate, caproate and branched SCFA (isobutyrate, isovalerate and isocaproate) were measured as described previously (De Weirdt et al, 2010).

**Extraction of bacterial DNA**

Before extraction of the mucosal samples, the samples were heated for 15 min at 55$^{o}$C to make the agar soluble. Subsequently, DNA was extracted from thawed samples using the QIAamp DNA Stool mini kit (Qiagen, Hilden, Germany) with a bead beater step in advance, as previously described (Leser et al, 2000). For each sample, DNA was extracted in duplicates. The purified DNA was stored at -20°C until use.

**PCR amplification for DGGE**

Aliquots (10 µL) of purified DNA (5 ng/µl of pooled DNA from the duplicate DNA extractions) were applied to the following to give a 50 µL PCR reaction mixture: 20 µL of 5 PRIME MasterMix (2.5×) (VWR & Bie & Berntsen) and 10 pmol of each of the primers (Eurofins MWG Synthesis GmbH, Ebersberg, Germany). Primers HDA1-GC/HDA2 (Walter et al, 2000) targeting 16S rRNA genes from all bacteria were used in a touchdown PCR as previously described (Petersen et al, 2010)

**Analysis of luminal and mucosal microbiota by DGGE**

DGGE was carried out as described previously (Bernbom et al, 2006) using a DcodeTM Universal Mutation Detection System instrument and gradient former model 475 according to the manufacturer's instructions (Bio-Rad Labs, Hercules, California). The 9% polyamide gels were made with denaturing gradients ranging from 25% to 65%. The 100% denaturant solution contained 40% formamide and 7M urea. Thirteen microlitres PCR products were mixed with 3 µL loading dye before loading. Gels were run in 1 x TAE at 60 °C for 16 h at 36 V, 28 mA, stained with ethidium bromide for 15 min, destained for 20 min, and viewed by UV-B trans illumination at 302 nm (Bio-Rad). The BioNumerics software, version 4.60 (Applied Maths, Sint-Martens-Latem, Belgium) was used for identification of bands and normalization of band patterns from DGGE gels. DGGE gels were normalized by an assigned marker (developed in our laboratory). A cluster analysis was performed based on Dice coefficient of similarity (weighted) using the unweighted pair group method and the arithmetic averages clustering algorithm.

**Quantitative PCR assay conditions**

QPCR was performed on an ABI Prism 7900 HT from Applied Biosystems. The amplification reactions were carried out in a total volume of 11 µL containing; 5.50 µL (SYBR® Green, Applied Biosystems), primers (each at 200 nM concentration) (Eurofins MWG Synthesis GmbH), 2 µL template DNA, and Nuclease-free water (Qiagen) purified for PCR. The amplification was carried out as previously described (Vigsnaes et al, 2011). DNA (5 ng/µl) from the duplicate DNA extractions of each sample was used for the qPCR.

**Quantitative PCR primer and data handling**

The primers specific to regions of the 16S rRNA genes of 20 selected bacterial taxa are listed in Table S1 (supplementary data). The relative quantities of gene targets encoding gene sequences of the bacterial taxa were calculated using $2^{DeltaCt}$, assuming primer efficiency at 1.0. Delta Ct is the Ct-values of the bacterial target normalized against Ct-values of the total bacterial population in a sample. Ct is the threshold cycle calculated by

the ABI software (SDS version 2.2; Applied Biosystems, Foster City, California, USA) as the PCR cycle, where amplification signal exceeds the selected threshold value, also set by the ABI software. Prior to the quantification, standard curves were created using serial 10-fold dilutions of bacterial DNA extracted from one of the M-SHIME samples for all primer sets. Analysis of the standard curves allowed verification of PCR efficiency for the chosen PCR conditions. All primers were tested to confirm sensitivity and specificity using DNA from pure bacterial species (Table S2, supplementary data). The detection limit was set to 0.001% of the quantity of the total bacteria. Bacterial targets that could not be detected or were below the detection limit were set to one half of the detection limit for further calculations.

**Metabolite detection & identification**

Luminal samples from the M-SHIME were thawed on ice and subsequently centrifuged at 3000 g for 15 min at 4°C. Cold methanol (around -80°C) in a ratio 1:1 was added to the supernatant. Metabolites from the mucosal samples, due to the semi-solid form, were extracted with 2 ml of cold 50% methanol and centrifuged at 3000g for 15 min at 4°C. This procedure was repeated twice. All of the samples were stored at -80°C until further analysis and centrifuged at 15000g for 5 min at 4°C just before LCMS analysis. The analysis of metabolites was conducted as follow: a Dionex Ultimate 3000 RS liquid chromatigraph (Dionex, Germering, Germany) coupled to a Bruker maXis time of flight mass spectrometer equipped with an electrospray interphase (Bruker Daltonics, Bremen, Germany). Analytes were separated on Kinetex pentafluorophenyl column 50 x 2.10 mm, 2.6 µm, 100Å (Phenomenex, USA), using the solvent system: A, 10 mM ammonium formate pH 3.5, and B, acetonitrile. Solvent programming was isocratic 0% B to 0 min followed by a linear gradient to 100% B at 7 min and 100% B at 8 min. Flow rate was 0.25 ml/min at 0 min, increased to 0.4 ml/min at 7 min. Solvent composition and flow were returned to initial conditions at 8.2 min. The oven temperature was 40°C. Injection volumes were 1 µl. The following electrospray interphase settings were used: nebulizer pressure 2 bar, drying gas 10 l/min, 200°C, capillary voltage 4000V. Scan range was from 50 to 800 m/z. The main focus of this study was acids, thus samples were analyzed in negative mode.

To identify metabolites seen to separate given groups, the Human Metabolome Database (HMDB; Wishart et al. 2009) was used. Molecular formulas of the metabolites from the LCMS analysis were generated based on exact mass and isotopic pattern with Bruker Daltonics Software. Identification of metabolites was based on a very low measurement error (Tab.1, Tab.2; external & internal calibration and lock mass was used), compared to the data given in HMDB and further references to the microbial metabolism in the GI. In addition hydrophobic properties of the molecule, indicated by the retention time in the chromatograph, were taken into consideration.

**Statistical analysis**

Statistical analysis of the qPCR data was performed using OriginPro software (version 8.1; OriginLab Corporation, Northampton, USA). Normality and homogeneity of variances of the qPCR data were assessed using the Kolmogorov-Smirnov and the Levene's test, respectively. Log-transformations were performed for data that did not meet these criteria. T-testing was apllied to identify significant differences in colonization ability using the ratio (proportion) of the given bacterial taxon present in the two communities (lumen and mucus) for the three groups (healthy, UC patients in remission and UC patients in relapse). Univariate ANOVA was used to determine significant differences between specific bacterial taxa comparing the three groups either from lumen or mucus. Where ANOVA indicated a significant difference, Fisher's least significant different test was used. The nonparametric Kruskal-Wallis test was used for datasets, which did not have homogeneity of variance or were not normally distributed even after log-transformation. Univariate ANOVA was applied to confirm that there was no age differences between the three disease groups. Tests were considered statistically significant if P-values lower than 0.05 were obtained. Principal Component Analysis (PCA) of qPCR data was carried out using LatentiX® data analytical software (version 2.00, The Mathworks Inc., Copenhagen, Denmark). Differences in metabolite profiles were evaluated by PCA using Profile Analysis 2.0 by Bruker Daltonics. Data was grouped into buckets of 1 min and 1 m/z differences in the range from 0.5 to 9 min and 50 to 800 m/z and normalized by the sum of buckets in the analysis. P-values were calculated by univariate ANOVA ($\alpha = 0.05$) using the normalized values from the PCA. As for the qPCR analysis, tests were considered significant when P-values lower that 0.05 were obtained.

## Results

### Microbial activity in terms of SCFA production

SCFA were analyzed in the luminal content of the colonic M-SHIME vessels as a measure of the metabolic activity of the microbiota derived from healthy subjects or UC patients, either in remission or relapse (Table 1). Forty-two hours after inoculation with fecal samples, there was a significant inter-individual variability within each group regarding the conversion of the provided nutrients to SCFA ($P<0.05$). As a result of this variability, no significant differences were detectable between the three groups. However, there was a trend of lower acetate and higher branched SCFA concentrations for UC patients as opposed to healthy subjects ($P=0.138$ and $P=0.210$, respectively). Additionally, we found it noteworthy that the levels of carproate found in samples containing microbiota from patients in remission was 36 fold higher than in samples with microbiota from patients in remission ($P=0.229$).

### Microbial community analysis using DGGE

Comparison of DGGE profiles containing 16S ribosomal genes amplified from luminal and mucosal samples of healthy subjects and UC patients after 42h colonization revealed a distinct difference between the dominant bacterial members of the luminal and mucosal environment (Figure 1). The dendrogram from the Dice cluster analysis showed three clusters with five luminal samples in cluster I (53.89% similarity), all mucosal samples in cluster II (54.61% similarity) and seven luminal samples in cluster III (41.15% similarity). Clustering of the distribution of these dominant microbial species did not correlate to the health status of the human subjects (healthy, UC in remission and UC in relapse).

### Microbial community analysis by qPCR

For microbiotas derived from UC patients in relapse or remission, luminal and mucosal samples were clearly separated from one another, while this separation was not equally

clear for samples from healthy subjects (Figure 2, score plot). Especially the second principal component (PC2) explained the difference between the luminal and mucosal environments with *Roseburia, Faecalibacterium prautznitzii*, and *Closteridiaceae/Eubacterium* representing the mucosal environment and *B. bifidum, B. adolescentis*, *Bifidobacterium* spp., *Lactobacillus* spp., *Akk. muciniphila* and *Actinobacteria* representing the luminal content (Figure 2, loading plot).

It was noteworthy that the preference of specific bacterial groups to colonize the mucosal and/or luminal compartment was clearly affected by the origin of the microbiota (healthy subjects, UC patients in remission or UC patients in relapse) (Table 2). No significant differences between the luminal and mucosal samples with respect to proportions of specific bacterial taxa were measured when fecal communities were derived from healthy subjects. However, in the vessels with communities derived from UC patients in relapse, mucus was colonized by significantly lower proportions of bifidobacteria, *B. bifidum,* lactobacilli, *C. coccoides* group, *C. leptum* subgroup and *Alistipes* spp.(P=0.01, P=0.02, P=0.03, P=0.05, P=0.02, and P=0.05, respectively) than found in lumen. Additionally, the densities of bifidobacteria, *B. adolescentis*, B*. pseudocatenulatum*, lactobacilli, *C. leptum* subgroup, *Faecalibacterium prausnitzii* and *Actinobacteria* derived from UC patients in remission were significantly lower in mucus than in lumen (P=0.01, P=0.001, P=0.01, P=0.007 and P=0.05, P=0.03 and P=0.001, respectively). Finally, significantly higher mucosal counts of *Roseburia* spp. derived from UC patients in remission were measured (P=0.05)

Comparison of the proportions of specific taxa present in the luminal and mucosal compartments, respectively, from the three types of microbiota (healthy, UC remission, UC relapse) revealed that relative quantities of *Bifidobacterium* spp. and *Lactobacillus* spp. in mucus was significantly lower (P=0.05 and P=0.002, respectively) in communities derived from UC patients in relapse than in those derived from healthy subjects, whereas the relative quantity of *Clostridiaceae/Eubacterium* was significantly higher in the lumen (P=0.04) in communities from UC patients in relapse than in those from healthy subjects (Table 3).

The bacterial growth rate of selected bacterial taxa in the lumen was calculated from the slope of the exponential phase of the growth curves. The growth rate was increased during

the 42 h incubation in the lumen for all the examined bacterial taxa. No significant difference in the growth rate for each bacterial taxon was found when comparing the three groups (healthy subjects, UC in remission or UC in relapse) (Table S3, supplementary data).

**Metabolite detection and separation**

PCA of the metabolites as detected by LCMS revealed a difference between samples taken from healthy subjects and UC patients in relapse, respectively. The grouping was present in samples from mucus (Figure 3, score plot) as well as from lumen (Figure 4, score plot). No difference was observed between UC patients in remission and healthy subjects (Figure S1 and S2, supplementary data). However, a clear separation between score plots for UC patients in relapse and remission, respectively, was seen (Figure S3 and S4, supplementary data).

Based on the PCA loading plots (Figure 3 and 4), metabolites, which were tentatively causative for the difference observed between UC patients in relapse and healthy subjects, were identified (Table 4 and 5). Loadings, which in extracted ion chromatograms showed clear chromatogram peaks (data not shown) that were present in significantly different levels ($P<0.05$) in the two groups were further investigated. Substances in the Human Metabolome Database, which corresponded to the found mass/charge ratio ($M_{LCMS}$) were identified. However, a few of the given mass/charge values had more than one possible match (Table 4 and 5).

## Discussion

In this study, we applied a recently developed dynamic *in vitro* gut model, the M-SHIME, to investigate differences between the intestinal microbial ecosystems of healthy subjects and UC patients, either in relapse or in remission. This model allows investigation of differences within the luminal content as well as at the artificial intestinal mucosal surface (Van den Abbeele et al, 2011a; Van den Abbeele et al, 2010). The impact of the human host on the microbial composition is eliminated, thus allowing focus on intrinsic features of the gut microbial populations.

In line with recent *in vivo* (Hong et al, 2011; Nava et al, 2011) and *in vitro* studies (Van den Abbeele et al, 2012; Vermeiren et al, 2012), we have demonstrated that also in the M-SHIME model, the in vitro mucosal microbial community differs from the luminal one (Figure 1), with specific butyrate-producing bacteria (e.g. Roseburia spp.) being abundant members of the mucosal microbiota (Table 2 and Figure 2). The luminal and mucosal bacteria have previously been demonstrated to display different roles in the host, and it has been proposed that the mucosal microbiota is more involved in interaction with the epithelial and immune cells than the luminal microbiota, because it resides closer to the intestinal epithelial cells (Derrien et al, 2011; Van den Abbeele et al, 2011b). Hence, an altered mucosal microbial community may play an important role in dysregulated immune responses.

For samples from UC patients a significantly lower proportion of lactobacilli and bifidobacteria residing in the mucus-microcosm than in the lumen was observed, while this was not the case for healthy microbiotas (Table 2). Notably, this was observed for UC patients in remission as well for those in relapse, indicating that the impaired ability of the lactic acid bacteria to adhere to mucus is present also when UC patients are free of severe symptoms. However, when directly comparing 'mucosal' populations from M-SHIME colonized with microbiota from UC patients to those colonized with healthy microbiotas, only patients in relapse had a significantly lower amount of bifidobacteria and lactobacilli than found in the 'healthy' samples (Table 3). This could be due to the fact that microbiota from UC patients in relapse in general contained lower amounts of lactic acid bacteria than microbiota derived from either healthy people or UC patients in remission. Species or strain specific mucus adhesion promoting proteins have been reported in several

bifidobacteria and lactobacilli (Gilad et al, 2011; Kankainen et al, 2009; Kleerebezem et al, 2010; Pretzer et al, 2005; Pridmore et al, 2004; Van Tassell & Miller, 2011). The expression of adhesion molecules may be changed in the lactic acid producing bacteria derived from UC patients, hence their inability to colonize the mucus *in vitro.* However, we would expect that host-induced differences in bacterial gene expression profiles would no longer prevail after 42 hours in the gut model. Alternatively, an altered composition of the species of lactobacilli and bifidobacteria derived from UC patients may explain the decreased ability of these groups to adhere to the mucin-microcosms of the M-SHIME. A third explanation may be that within a given species, the strains of lactic acid bacteria present in UC patients are less capable of adhesion to mucins than their counterparts present in healthy subjects, perhaps due to minor genetic differences in adhesion-relevant genes.

Bifidobacteria and lactobacilli are believed to play important roles in promoting intestinal health (Chenoll et al, 2011; Collado et al, 2007; Fooks &.Gibson, 2002; Hoarau et al, 2006; Lee et al, 2011; Lee et al, 2003; Saulnier et al, 2011; Zeuthen et al, 2010). The observed depletion of bifidobacteria and lactobacilli in UC patients in relapse, which is in line with a number of previous reports (Macfarlane et al, 2004; Mylonaki et al, 2005; Vigsnaes et al, 2012), is likely to have a consequence for colonic health. It is, however, not possible from the given data to conclude whether the impaired adhesion capacity of lactic acid bacteria derived from UC patients is part of the cause or part of the effect of the disease. It may be that the mucus layers in the intestines of the UC patients have selected for populations of lactic acid bacteria, which are less optimized to adhere to the 'healthy' mucin of the M-SHIME.

Also the ability of the butyrate-producing clostridial groups *C. coccoides* and *C. leptum* to colonize the mucin-covered microcosms of the M-SHIME was found to be lower in bacterial communities from UC patients than in those originating from healthy subjects (Table 2). This may be linked to the lower abundance of lactobacilli and bifidobacteria in the microcosms, since these species produce acetate and/or lactate, which is subsequently utilized by the given clostridial groups. In contrast to the *C. coccoides* group and the *C. leptum* subgroup, the buturate-producing *Roseburia* spp. in microbiotas originating from UC patients demonstrated a high presence mucin-microcosms (Table 2),

indicating that these species, which are known to be able to degrade complex carbohydrates (Flint et al, 2007), are not dependent on the presence of lactate and acetate as seen for their clostridial relatives. Hence, the low abundance of the clostridial groups within the mucin-microcosms may create a free ecological niche allowing for excessive colonization of *Roseburia* spp.

Metabolomic (LCMS) analysis revealed that the microbiotas from healthy subjects and from UC patients in remission displayed very similar metabolisms, while the metabolism of bacterial communities from UC patients in relapse was clearly different from these two groups (Figure 3, 4, S1, S2, S3 and S4). We found that metabolism of bile acids, tryptophan and phenylalanine were altered in luminal as well as mucosal samples derived from UC patients in relapse as compared to healthy subjects (Table 4 and 5). The presence of drugs and drug metabolites in samples from UC patients in relapse was expected, since all four patients received mesalazine (5-aminosalicylic acid).

In the human body, most bile acids are actively reabsorbed from the ileum and returned to the liver. However, a small fraction of bile acids escape enterohepatic circulation and enter the colon where bacteria metabolize the bile acids primarily by deconjugation and oxidation of hydroxyl groups. Bile salt hydrolysis is carried out by a broad spectrum of intestinal bacteria (Jones et al, 2008; Ridlon et al, 2006). However, the specificity of hydroxysteroid dehydrogenase (HSDH) varies depending on bacterial species and may either be specific for the 3-, 7-, and/or 12-hydroxy groups of bile acids leading to different secondary bile acids. Specific HSDHs have primarily been detected in a number of *Clostridium* and *Eubacterium* species belonging to either cluster I, IV (*C. leptum* subgroup), XIVa (*C. coccoides* group) or XIb (Doerner et al, 1997; Ridlon et al, 2006). As we demonstrated significantly higher levels of *Clostridiaceae/Eubacterium* in the luminal samples from UC patients in relapse than in healthy subjects (Table 3), this could partly explain the observed enrichment of secondary bile acids. High levels of secondary bile acids such as deoxycholic acids (primarily produced by species belonging to *Clostridiaceae*) can be detrimental for colon health (Mcgarr et al, 2005).

Phenylalanine was more abundant in mucosal samples from the M-SHIME colonized with microbiota from healthy subjects, than in those colonized with samples from UC patients (Table 4). Products of phenylalanine that are normally metabolized by intestinal bacteria undergo a variety of processes in the body, where they may be detoxified by either

glucuronide or sulphate conjugation in the gut mucosa and liver, or remain unabsorbed and voided in feces (Smith & Macfarlane, 1997). Production of phenolic compounds by proteolytic and peptidolytic activities of the intestinal bacteria has previously been associated with various diseases including cancer and UC (Smith &.Macfarlane, 1997). Previous studies have shown that species belonging to the genera *Clostridium* and *Bacteroides* have a high capacity to ferment phenylalanine to phenolic compounds such as phenylpropionate, phenylacetate and/or phenyllactate (Elsden et al, 1976; Smith &.Macfarlane, 1996). Here, we observed higher levels of clostridia in samples derived from UC patients than in samples from healthy subjects (Table 3). Hence, we speculate that the enrichment of products of phenylalanine metabolism in mucosal and luminal samples derived from UC patients in relapse could be a result of the higher abundance of these bacterial groups (Table 4 and 5).

Also tryptophan levels were higher in samples from the M-SHIME colonized with microbiota from healthy subjects than when it was colonized with microbiota from UC patients in relapse (Table 4 and 5). In line with this, previous studies have revealed that after fermentation using inocula from healthy subjects, no products of tryptophan metabolism could be detected in the system (Smith &.Macfarlane, 1996; Smith &.Macfarlane, 1997).

Finally, we observed that unsaturated fatty acids (FA) were significantly higher in mucus compartments colonized with bacteria from UC patients in relapse than in those colonized with 'healthy' bacterial communities (Table 4). Previous metabolomic research has revealed changes of FA in serum of UC patients (Hengstermann et al, 2008; M.Esteve, 2004), and has suggested that these changes could be caused by increased endogenous biosynthesis of polyunsaturated fatty acids (PUFA) and by increased lipolysis initiated by cytokines during the inflammatory response. Based on the present work, we speculate that the altered levels of FA in serum UC patients in relapse compared to healthy subjects could be originating from the intestinal microbial metabolism.

In conclusion, our data suggest that the significantly altered intestinal bacterial community present in UC patients in relapse results in a significantly altered bacterial metabolic profile. The observed bacterial alterations are suggested to result in increased metabolism of phenylalanine and tryptophan in microbial communities from UC patients in relapse.

While UC patients in remission display microbiotas and metabolomes very similar to those of healthy subjects, the lactic acid bacteria present in patients in remission are, similarly to those from the relapse samples, significantly impaired in their ability to adhere to the mucus microcosms of the M-SHIME. This may be due to a different composition or a different genetic makeup of the lactic acid bacteria present in all UC patients, which may play a role in the etiology of this disease. We suggest that probiotic therapy for UC patients should not exclusively aim at increasing the amount of lactic acid bacteria present in the gut, which has previously proved helpful (Zocco et al, 2006), but also at replacing the existing lactic acid bacteria with other strains/isolates with better capacity for mucosal adhesion.

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## Tables and figures

**Table 1** The average (± SEM) absolute SCFA levels (mM) in the luminal content of the M-SHIME units, 42h after inoculation with fecal samples of different human subjects: healthy, UC remission and UC relapse (n = 4).

| | **Healthy** | **UC remission** | **UC relapse** |
|---|---|---|---|
| Acetate | 54.0 ± 3.8 | 40.7 ± 4.9 | 46.1 ± 6.0 |
| Propionate | 7.3 ± 0.3 | 6.2 ± 3.2 | 9.4 ± 1.4 |
| Butyrate | 23.6 ± 5.3 | 18.9 ± 4.2 | 23.8 ± 3.4 |
| Valerate | 0.6 ± 0.6 | 1.6 ± 0.8 | 0.7 ± 0.6 |
| Caproate | 1.5 ± 1.5 | 3.6 ± 2.8 | 0.1 ± 0.1 |
| Branched SCFA | 2.0 ± 1.6 | 5.1 ± 1.2 | 3.2 ± 1.3 |
| Total SCFA | 89.1 ± 2.5 | 76.2 ± 4.6 | 83.3 ± 7.6 |

**Table 2** Preference of bacterial taxa to colonize the mucosal compartment, expressed as ratio of the relative quantities in mucosal and luminal compartments of the M-SHIME.

| | Ratios mucus/lumen (%)[a] | | |
|---|---|---|---|
| **Bacterial taxa** | **Health subjects** | **UC remission** | **UC relapse** |
| *Firmicutes* | 100.36 (±1.23) | 100.46 (±1.64) | 99.85 (±0.89) |
| • *Clostridiaceaec*[b]*/Eubacterium* | 103.18 (±29.64) | 117.47 (±19.49) | 79.53 (±7.99) |
| • *Clostridium leptum* subgroup | 96.33 (±8.43) | 83.81 (±5.12)* | 82.39 (±3.35)* |
| ○ *Faecalibacterium prausnitzii* | 102.62 (±13.35) | 83.75 (±4.22)* | 93.91 (±4.97) |
| • *Clostridium coccoides* group | 88.47 (±10.94) | 74.10 (±11.74) | 66.81 (±10.73)* |
| ○ *Roseburia* spp. | 108.01 (±7.70) | 137.83 (±12.28)* | 146.06 (±41.24) |
| • *Lactobacillus* spp. | 94.70 (±8.85) | 81.32 (±2.77)** | 68.59 (±7.64)* |
| *Bacteroidetes* | 99.98 (±2.84) | 106.52 (±9.69) | 94.36 (±7.57) |
| • *Bacteroides* spp. | 98.44 (±2.87) | 112.93 (±9.33) | 85.28 (±5.06) |
| ○ *Bac. fragilis* group | 93.95 (±7.55) | 107.24 (±14.33) | 75.48 (±8.88) |
| • *Alistipes* spp. | 65.05 (17.58) | 68.12 (±11.76) | 71.91 (±8.47)* |
| *Actinobacteria* | 76.29 (13.79) | 55.39 (2.88)*** | 52.01 (16.96) |
| • *Bifidobacterium* spp. | 94.60 (±3.12) | 91.24 (±1.53)** | 84.63 (±4.28)** |
| ○ *B. bifidum* | 93.99 (±3.47) | 106.37 (±6.97) | 84.48 (±3.35)* |
| ○ *B. adolescentis* | 89.52 (±4.61) | 83.68 (±1.37)*** | 67.61 (±16.58) |
| ○ *B. pseudocatenulatum* | 101.27 (±4.68) | 79.79 (±3.55)** | 94.60 (±15.00) |
| *Proteobacteria* | | | |
| • *Desulfovibrio* spp. | 101.19 (±1.75) | 101.22 (±4.53) | 92.74 (±3.89) |
| *Verrucomicrobia* | | | |
| • *Akkermansia muciniphila* | 85.48 (±6.90) | 73.15 (±12.19) | 86.18 (±5.10) |

[a] Ratios (%) calculated as 100*mucosal samples/luminal samples, hence values below 100% indicates low adherence capacity to mucus, while values above 100% indicate high adherence capacity to mucus.
[b]Includes Clusters I, III, IV, XIVa, XIVb
Asterisks (*) indicate significant differences between mucosal and luminal samples (*P<0.05 and **P<0.01). All calculated data are means ± SEM.

**Table 3** Relative abudance of bacteria derived from UC patients compared to healthy subjects

| | Lumen | | Mucus | |
|---|---|---|---|---|
| **Bacterial taxa** | **UC remission** | **UC relapse** | **UC remission** | **UC relapse** |
| *Firmicutes* | 98.99 (±2.34) | 101.92 (±0.52) | 99.02 (±0.96) | 101.43 (±0.40) |
| • *Clostridiaceaec*[a]*/Eubacterium* | 111.60 (±13.44) | 142.63 (±8.74)* | 131.70 (±9.89) | 120.27 (±13.98) |
| • *Clostridium leptum* subgroup | 105.94 (±9.04) | 120.45 (±3.06) | 95.21 (±10.49) | 106.03 (±4.53) |
| ○ *Faecalibacterium prausnitzii* | 120.28 (±7.78) | 125.79 (±3.57) | 103.39 (±2.32) | 122.12 (±5.42) |
| • *Clostridium coccoides* group | 97.68 (±14.02) | 119.86 (±6.22) | 69.89 (±23.95) | 94.02 (±16.97) |
| ○ *Roseburia* spp. | 103.12 (±7.65) | 72.29 (±11.66) | 133.15 (±19.83) | 89.96 (±24.74) |
| • *Lactobacillus* spp. | 109.74 (±3.52) | 80.06 (±12.88) | 96.27 (±4.42) | 57.99 (±8.93)*** |
| *Bacteroidetes* | 95.33 (±11.68) | 107.10 (±4.71) | 98.92 (±6.41) | 100.04 (±4.25) |
| • *Bacteroides* spp. | 93.35 (±11.66) | 110.06 (±6.83) | 104.27 (±6.83) | 94.82 (±6.63) |
| ○ *Bac. fragilis* group | 94.12 (±15.12) | 113.49 (±8.76) | 103.68 (±14.08) | 88.68 (±6.82) |
| • *Alistipes* spp. | 100.97 (±11.46) | 111.57 (±6.01) | 100.60 (±19.04) | 118.90 (±18.49) |
| *Actinobacteria* | 101.99 (±7.42) | 100.10 (±3.65) | 74.06 (±8.91) | 68.72 (±23.89) |
| • *Bifidobacterium* spp. | 99.97 (±3.72) | 97.65 (±2.65) | 96.53 (±4.95) | 89.04 (±4.22)* |
| ○ *B. bifidum* | 71.84 (±24.63) | 79.50 (±11.98) | 75.73 (±24.27) | 71.53 (±11.61) |
| ○ *B. adolescentis* | 112.86 (±4.80) | 89.80 (±15.22) | 106.56 (±5.15) | 76.15 (±23.01) |
| ○ *B. pseudocatenulatum* | 113.94 (±7.96) | 96.16 (±22.55) | 99.55 (±8.11) | 90.74 (±18.72) |
| *Proteobacteria* | | | | |
| • *Desulfovibrio* spp. | 117.09 (±17.88) | 120.72 (±5.61) | 117.92 (±18.33) | 111.03 (±4.90) |
| *Verrucomicrobia* | | | | |
| • *Akkermansia muciniphila* | 90.88 (±19.54) | 79.57 (±17.82) | 85.11 (±24.57) | 73.99 (±14.50) |

All numbers are average ± SEM of the four samples in each UC group of either lumen or mucus. The relative abundance values (%) are normalized to healthy controls (set to 100).
[a]Includes Clusters I, III, IV, XIVa, XIVb
Asterisks (*) designate a significant difference from the healthy control group (*P < 0.05 and ***P<0.001).

**Table 4** Annotated metabolites, which differ between healthy subjects and UC patients in relapse (mucus)

| *Enriched in UC relapse* | | | | | | |
|---|---|---|---|---|---|---|
| No. | Metabolite candidate | $M_{PCA}$ (Da)/ RT (min) | $M_{LCMS}$ (Da) | $M_{HMDB}$ (Da) | Error (mDa) | P values |
| 1 | Monounsaturated fatty acids (C18) | 281.5/6 | 281.248569 | 281.248627 | 0.058 | P<0.05 |
| 2 | Polyunsaturated fatty acids (C18) | 279.5/6 | 279.232812 | 279.232971 | 0.159 | P<0.05 |
| 3 | Secondary bile acids | 391.5/4 | 391.285401 | 391.285400 | 0.001 | P<0.05 |
| 4 | Salicyluric acid (drug) | 194.5/2 | 194.046042 | 194.045883 | 0.159 | P<0.001 |
| 5 | Monounsaturated fatty acids (C18) | 282.5/6 | 281.248569 | 281.248627 | 0.047 | P<0.05 |
| 6 | Aminosalicylic acid (drug metabolite) or 3-Hydroxyanthranilic acid (oxidation product of tryptophan metabolism) | 152.5/1 | 152.035358 | 152.035309 | 0.049 | P<0.01 |
| 7 | Product of phenylalanine metabolism | 149.5/3 | 149.060764 | 149.060806 | 0.042 | P<0.01 |
| *Enriched in healthy subjects* | | | | | | |
| No. | Metabolite candidate | $M_{PCA}$ (Da) | $M_{LCMS}$ (Da) | $M_{HMDB}$ (Da) | Error (mDa) | P values |
| 8 | Tryptophan | 203.5/2 | 203.082491 | 203.082596 | 0.105 | P<0.05 |
| 9 | Phenylalanin | 164.5/1 | 164.071619 | 164.071701 | 0.082 | P<0.005 |
| 10 | Hydroxyphenyllactic acid or 3-(3-hydroxyphenyl)-3-hydroxypropanoic acid | 181.5/2 | 181.050629 | 181.050629 | 0.531 | P<0.05 |

Numbers from 1-10 correspond to the metabolite marked in the loading plot Figure 3. $M_{PCA}$ designates mass taken from PCA; RT designates retention time bucket of the PCA; $M_{LCMS}$ designates mass taken from LCMS analysis of test samples; $M_{HMDB}$ designates mass given by Human Metabolome Data Base; 'Error' designates the mass difference between measured $M_{LCMS}$ and found $M_{HMDB}$.

**Table 5** Annotated metabolites, which differ between healthy subjects and UC patients in relapse (lumen)

| *Enriched in UC relapse* | | | | | | |
|---|---|---|---|---|---|---|
| No. | Metabolite candidate | $M_{PCA}$ (Da)/ RT (min) | $M_{LCMS}$ (Da) | $M_{HMDB}$ (Da) | Error (mDa) | P values |
| 11 | Secondary bile acids | 391.5/4 | 391.285527 | 391.285400 | 0.127 | P<0.01 |
| 12 | Salicyluric acid (drug) | 194.5/2 | 194.046042 | 194.045993 | 0.110 | P<0.001 |
| 13 | Aminosalicylic acid (drug metabolite) or 3-Hydroxyanthranilic acid (oxidation product of tryptophan metabolism) | 152.5/1 | 152.035358 | 152.035309 | 0.052 | P<0.05 |
| 14 | Product of phenylalanine metabolism | 149.5/3 | 149.060764 | 149.060806 | 0.122 | P<0.001 |
| *Enriched in healthy subjects* | | | | | | |
| No. | Metabolite candidate | $M_{PCA}$ (Da) | $M_{LCMS}$ (Da) | $M_{HMDB}$ (Da) | Error (mDa) | P values |
| 15 | Tryptophan | 203.5/2 | 203.082491 | 203.082596 | 0.278 | P<0.001 |
| 16 | Phenyllactic acid or 4-methoxyphenylacetic acid or desaminotyrosine | 165.5/3 | 165.055687 | 165.055710 | 0.023 | P<0.05 |
| 17 | Phenylglycine | 150.5/2 | 150.056153 | 150.056046 | 0.107 | P<0.001 |

Numbers from 11-17 correspond to the metabolite marked in the loading plot Figure 4. $M_{PCA}$ designates mass taken from PCA; RT designates retention time bucket of the PCA; $M_{LCMS}$ designates mass taken from LCMS analysis of test samples; $M_{HMDB}$ designates mass given by Human Metabolome Data Base; 'Error' designates the mass difference between measured $M_{LCMS}$ and found $M_{HMDB}$.

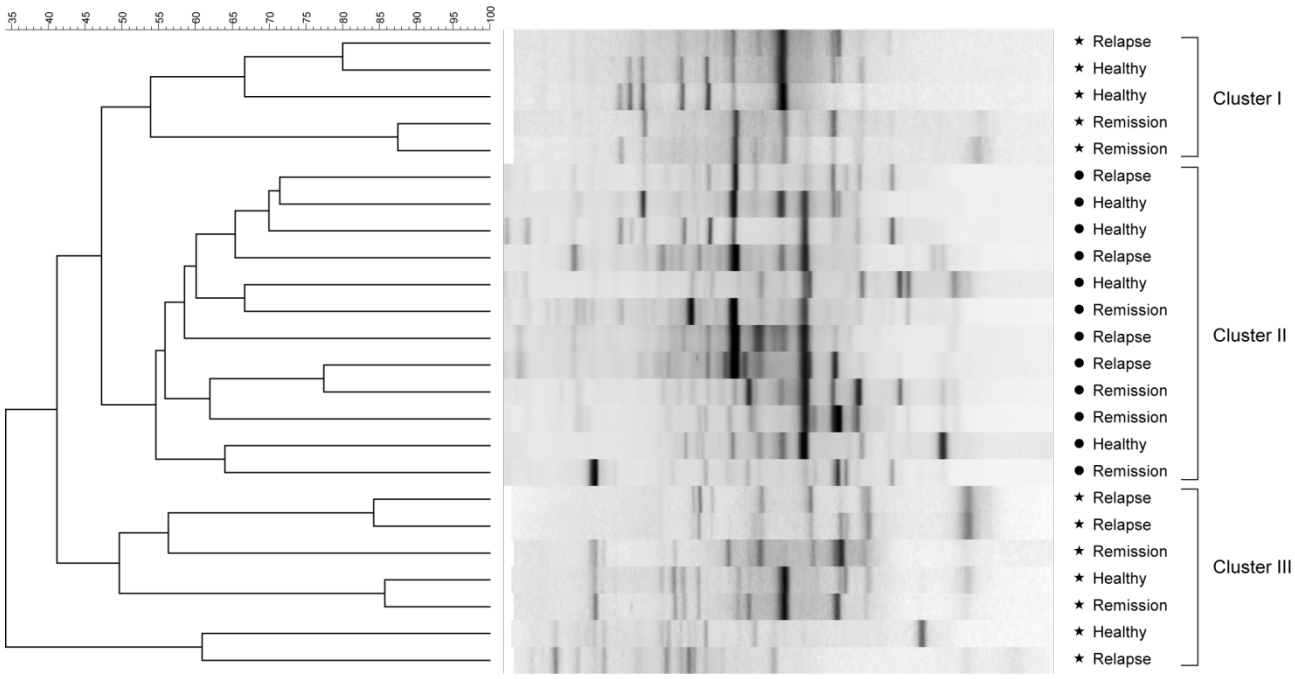


**Figure 1** Dice cluster analysis of universal DGGE gel profiles from *in vitro* luminal and mucosal communities of the M-SHIME colonized with samples derived from healthy subjects,or from UC patients in remission or relapse. The luminal samples are indicated by stars (*) and the mucosal samples are indicated by full circle (•). The dendrogram can be divided into three clusters: Cluster I. luminal samples (53.89% similarity). Cluster II. mucosal samples (54.61% similarity). Cluster III. luminal samples (41.15% similarity). Metric scale indicates degree of similarity in percentages.

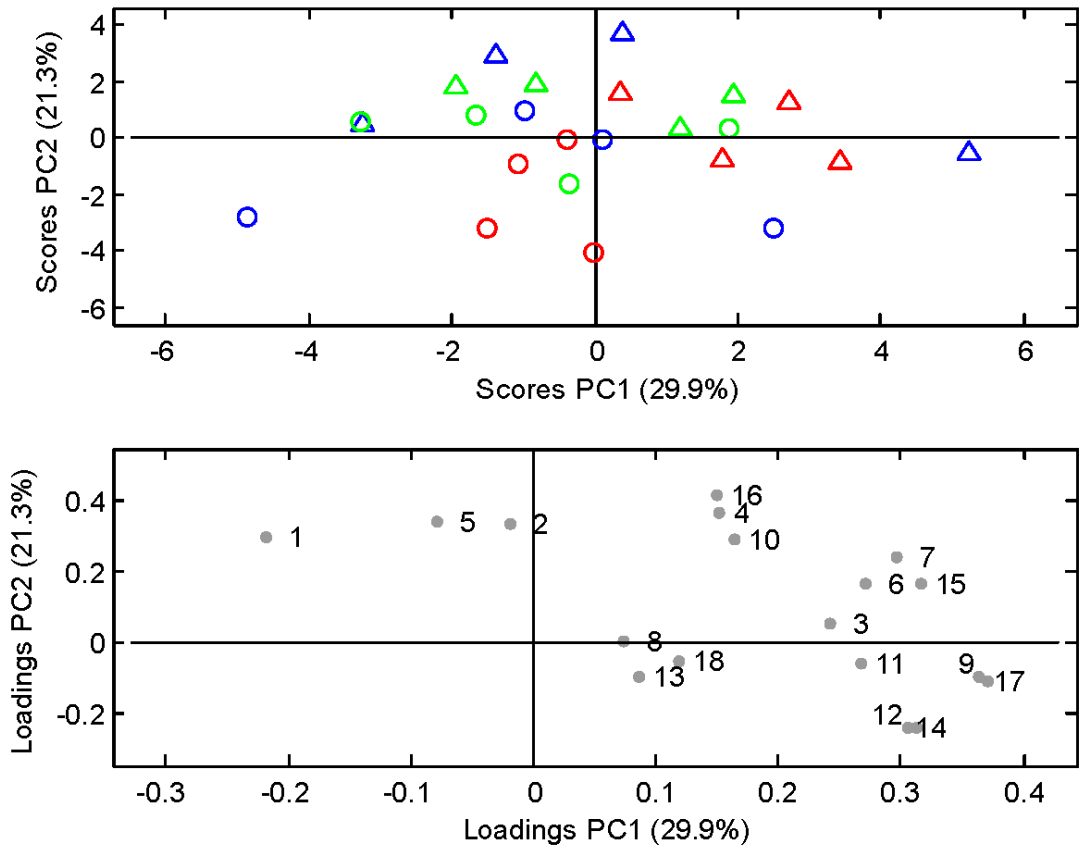


**Figure 2** Principal Component Analysis of the quantitative PCR measurements illustrated by. PC1 and PC2 (29.9% and 21.3% of explained variance, respectively). Score plot showing the M-SHIME luminal (Δ) and mucosal (o) communities. Sources of the communities are indicated by green for healthy subjects, blue for UC patients in remission and red for UC patients in relapse. Loading plot indicating each of the measured bacterial taxa as determined by quantitative Real-Time PCR. 1. *B. bifidum*; 2. *B. adolescentis*; 3. *B. pseudocatenulatum*; 4. *Bifidobacterium* spp.; 5. *Lactobacillus* spp.; 6. *C. leptum* subgroup; 7. *C. coccoides* group; 8. *F. prausnitzii*; 9. *Desulfovibrio* spp.; 10. *Akk. muciniphila*; 11. *Firmicutes*; 12. *Bacteroidetes*; 13. *Roseburia* spp.; 14. *Bacteroides* spp.; 15. *Alistipes* spp.; 16. *Actinobacteria*; 17. *Bac. fragilis* group; 18. *Clostridiaceae/Eubacterium*.

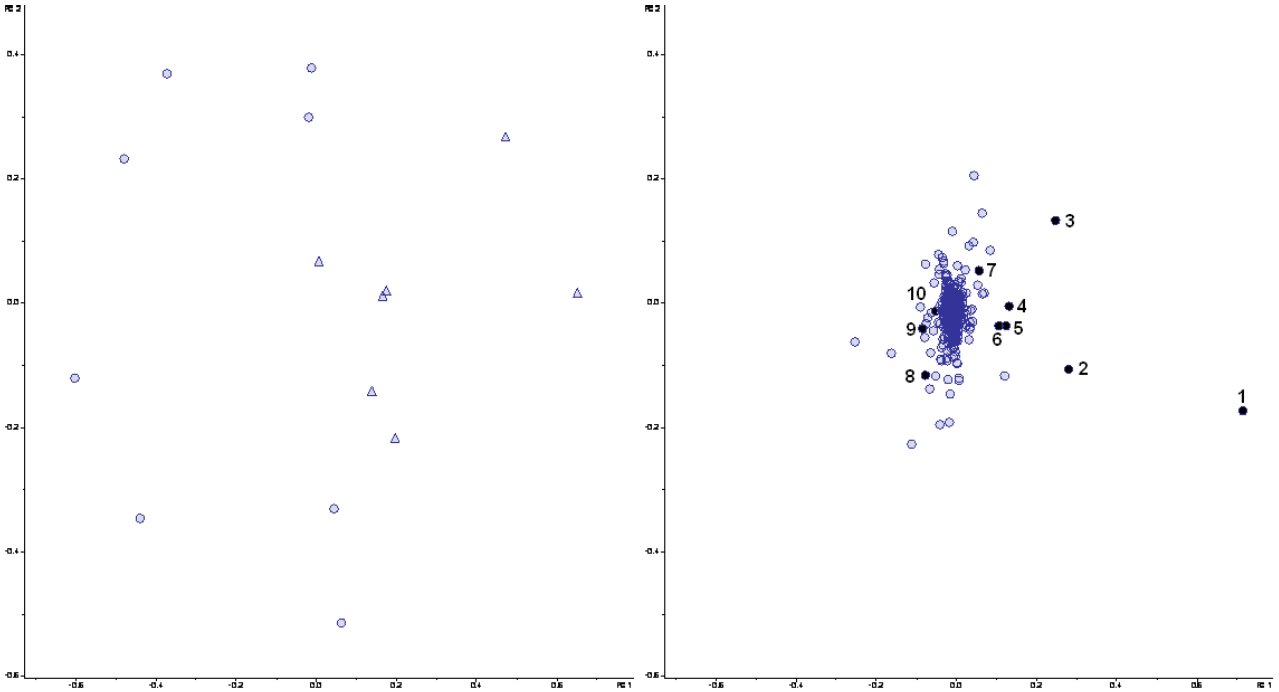


**Figure 3** Principal Component Analysis of LCMS data from the mucosal M-SHIME samples after colonization with microbiota derived from either healthy subjects (o) or UC patients in relapse (Δ). Score (left) and loading (right) plots are shown. PC1 and PC2 explain 29.1% and18.6% of the variance, respectively. Numbers from 1 to 10 in the loading plot correspond to metabolite candidates enriched in the M-SHIME mucin-coveres microcosms.. Masses of the candidates are given in Table 4.

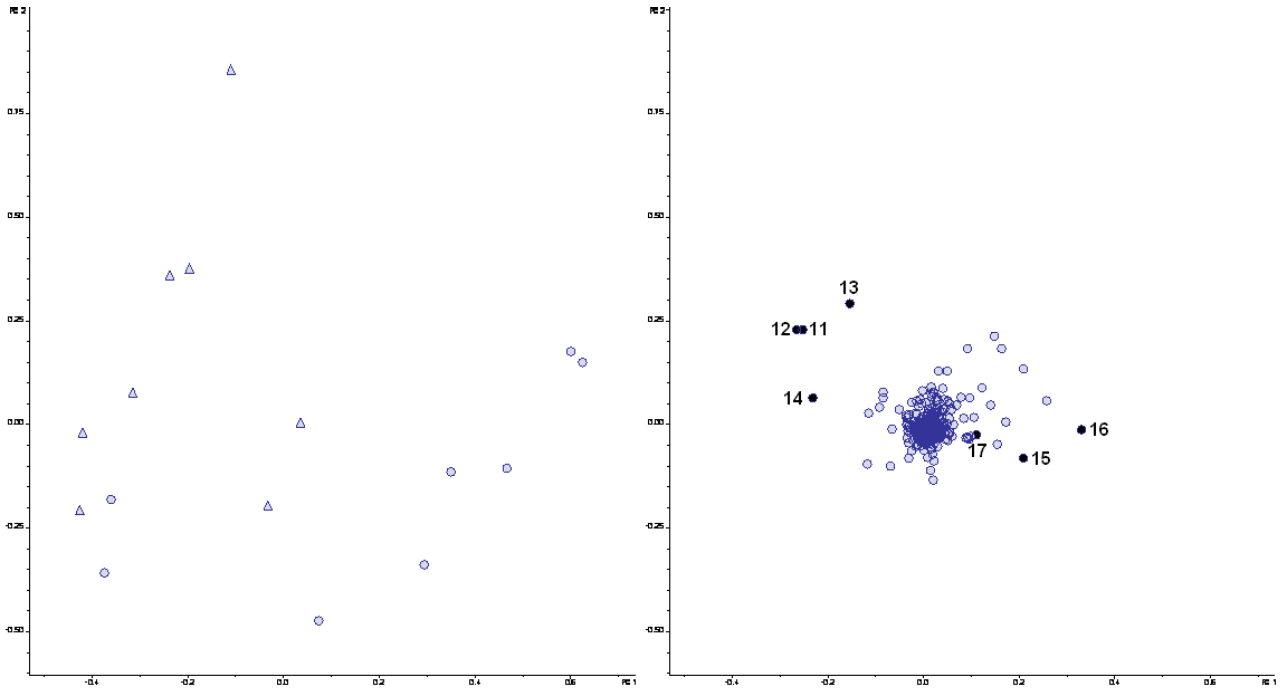


**Figure 4** Principal Component Analysis of the LCMS data from the luminal M-SHIME samples after colonization with microbiota derived from healthy subjects (o) or UC patients in relapse (Δ). Score (left) and loading (right) plots are shown. PC1 and PC2 explain 26.2% and 21.8% of the variance, respectively. Numbers from 11-17 in the loading plot correspond to metabolite candidates that are enriched in the luminal M-SHIME samples. Masses of the candidates are given in Table 5.

## Supplementary figures

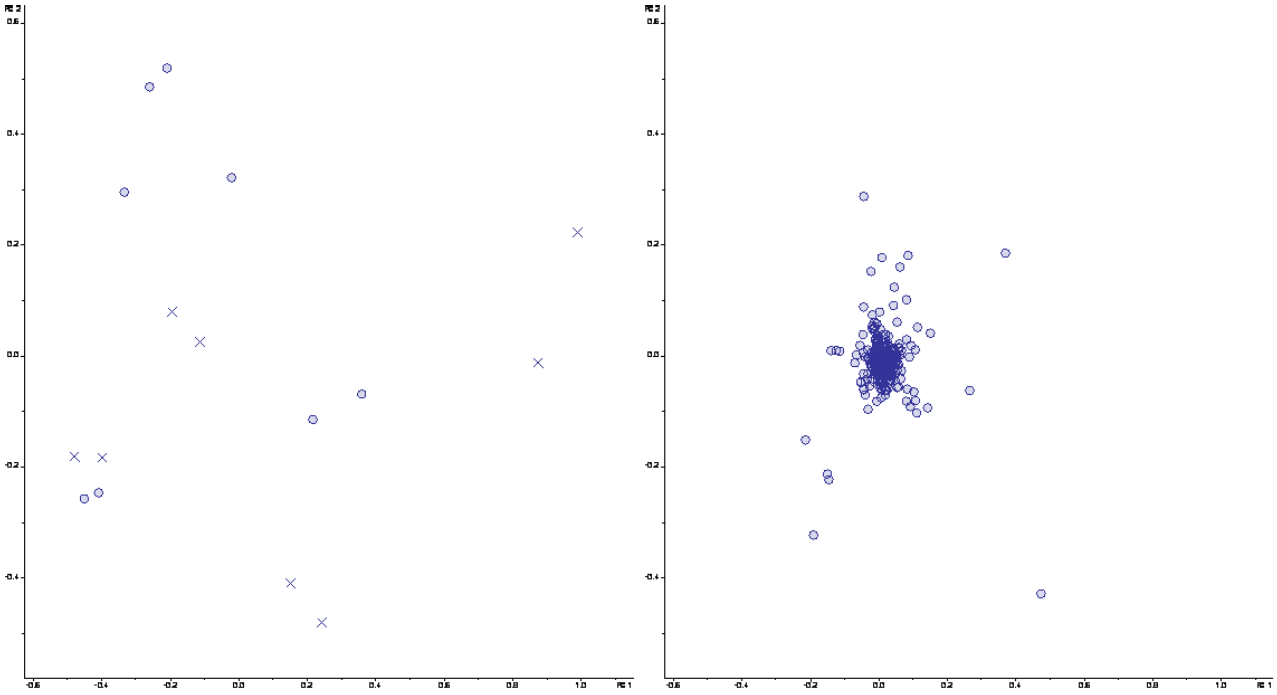

**Figure S1** PCA of LCMS data from mucosal samples of M-SHIME colonized with microbiota from healthy subjects (o) and UC patients in remission (x). Score (left) and loading (right) plots are shown. Presented PC1 (36.3%) vs. PC2 (16.4%).

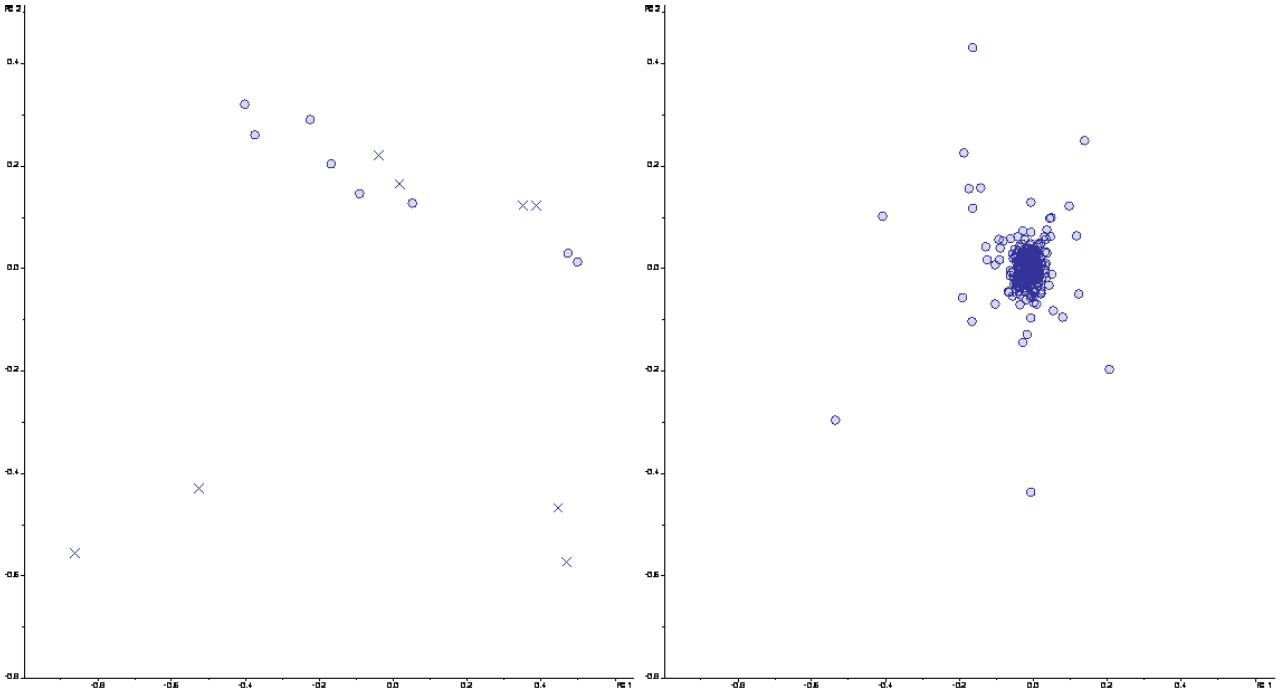

**Figure S2** PCA of LCMS data from luminal samples of M-SHIME colonized with microbiota from healthy subjects (o) and UC patients in remission (x). Score (left) and loading (right) plots are shown. Presented PC1 (30.3%) vs. PC2 (17.5%).

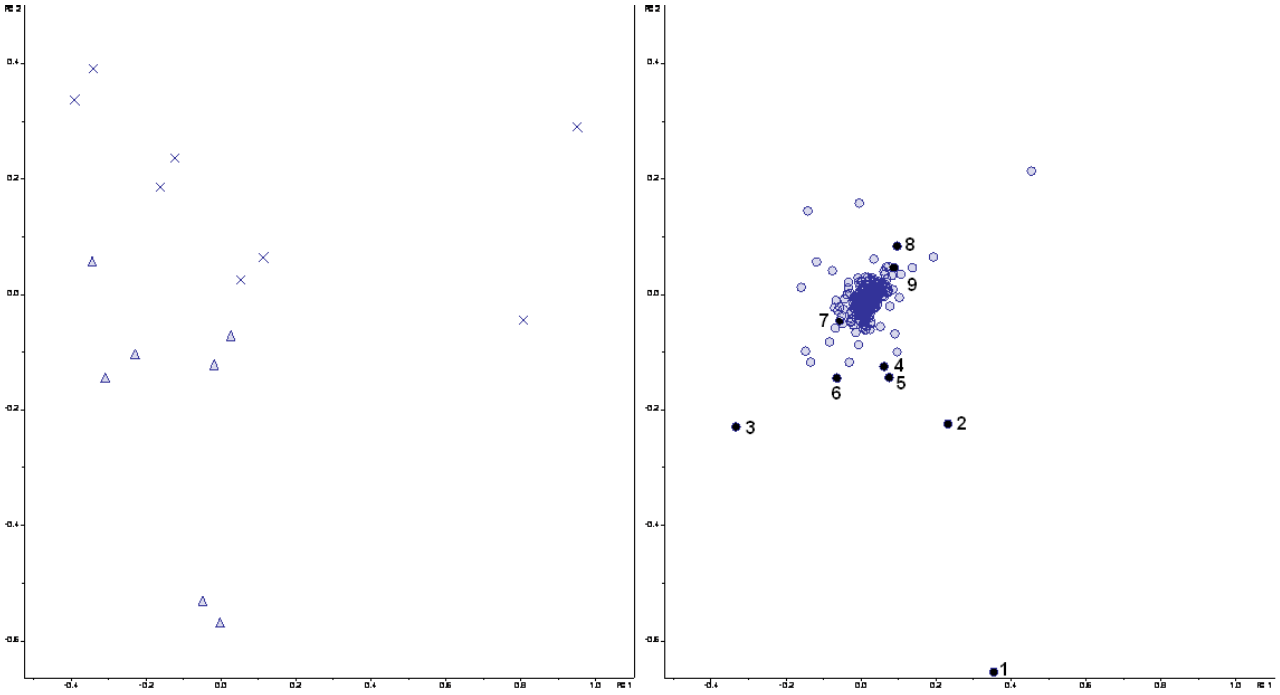


**Figure S3** PCA of LCMS data from mucosal samples of M-SHIME colonized with microbiota from UC patients in relapse (Δ) and in remission (x). Score (left) and loading (right) plots are shown. Presented PC1 (33.7%) vs. PC2 (17.5%). Numbers in the loading plot, bucket mass from the PCA /retention time bucket, correspond to the metabolite candidates described in Table 4.

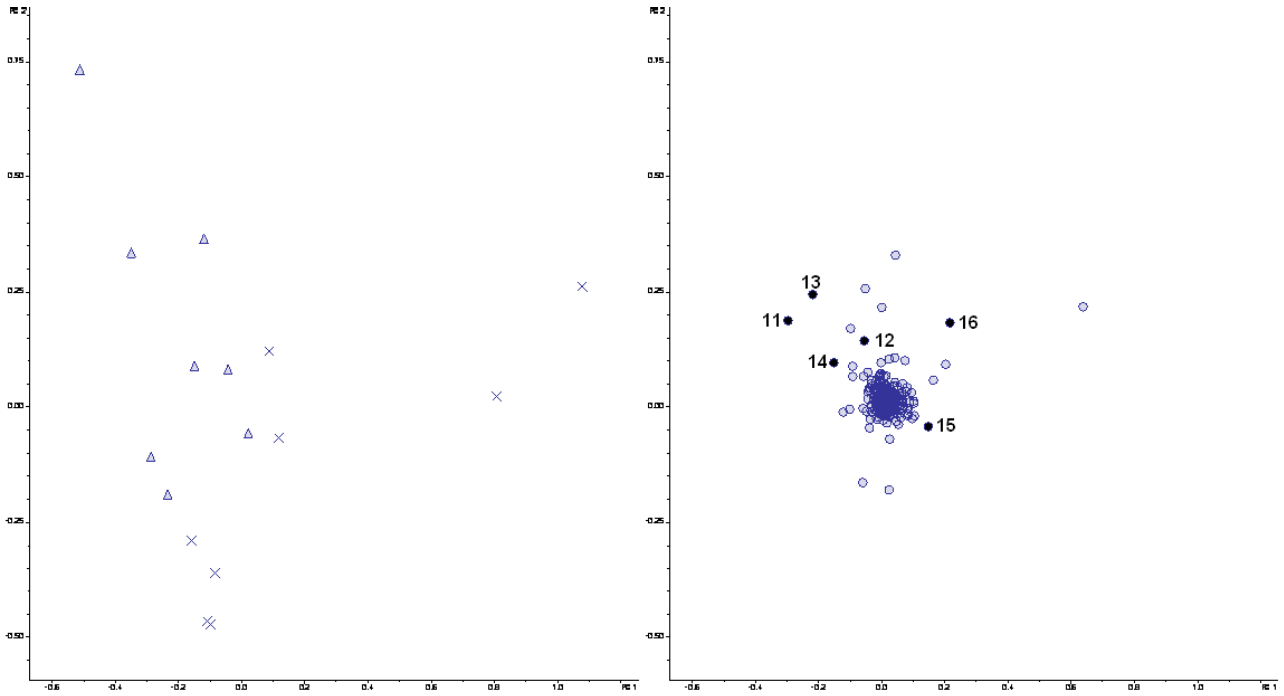


**Figure S4** PCA of LCMS data from luminal samples of M-SHIME colonized with microbiota from UC patients in relapse (∆) and in remission (x). Score (left) and loading (right) plots are shown. Presented PC1 (26.9%) vs. PC2 (17.4%). Numbers in the loading plot, bucket mass from the PCA /retention time bucket, correspond to the metabolite candidates described in Table 5.

# Bacterial Impact on the Gut Metabolome

# Discussion and final conclusions

# Discussion and final conclusions

The human gastrointestinal tract is a very complex system of interactions between microflora, diet and the host. Biochemical messages can be sent by many different types of molecules, including metabolites [199]. Metabolic footprinting represents only a small fraction of the whole metabolome; however it provides a key understanding of cell and organism communication mechanisms, which play a crucial role in the symbiotic relationships between GIT microflora and the host. In an extracellular environment any changes in the abundance and level of extracellular metabolites will directly reflect any modifications of the environment caused by activities of microorganism present in the system [18]. Due to the chemical and physical differences between various metabolites in the metabolome and due to the data overload, metabolomic studies are mainly focused on the differences between samples. Additionally, metabolic footprinting in the sample preparation is straightforward, when combined with the *in vitro* fermentation studies.

Therefore, in order to analyze the response of *Lactobacillus acidophilus* NCFM to a surrounding environment, starting pH of fermentations was set to 3, 5 and 7. As expected, NCFM did not grow in the low pH environment, but this has been used as a base to study the potential interactions of NCFM with the GIT surroundings at pH 5 and 7, as encountered throughout the human GIT. *In vitro* studies with NCFM showed that this strain in the presence of a simple carbohydrate source (glucose) increased the concentration of lactic acid, succinic acid, adenine and arginine in the medium after 24 h of fermentation, using adenosine and glucose as the primary source of energy. As an influence of pH on the enzymatic activities of NCFM was suggested, still pH 5 or 7 was not a strong indicator of the NCFM colonization and qualitative metabolite production. Lactic acid and succinic acid, produced by the probiotic strain could have a beneficial effect on the host, lowering the pH in the intestines and thereby protecting from pathogenic infections and cancer development [200-202]. However, the GIT is much more complex environment that is affected by nutrition available for the bacterial fermentation and mammalian metabolites interacting with the probiotic. Therefore, a simplified mammalian model, the germ-free mice studies, was introduced to analyze the NCFM influence on the host. Previous studies

using *Lactobacillus paracasei* NCC2461 [203] to colonize germ-free mice showed a great influence of this strain on the metabolic profiles of intestinal tissues, consistent with modulation of intestinal digestion, absorption of nutrients, energy metabolism, lipid synthesis and protective functions. Our initial analyses, comparing germ-free and monocolonized animals with NCFM, showed a distinctive differences in the metabolism throughout the mammalian GIT as well as global metabolism, represented by plasma and liver samples. The characteristics of NCFM influence on the host by metabolite identification and comparison to the previous *in vitro* fermentations still remain to be addressed. On the other hand, the mouse germ-free model in the evaluation of the probiotic effects will not present all of the NCFM interaction encountered in the mammalian GIT. Simplification by removal of other microbiota from the gut could obscure the real picture of NCFM influence on the host and that is why human baby flora mice [204] and human clinical trials in connection to the metabolomics should be considered in the future.

Metabolic footprinting was also introduced to the *in vitro* analysis of the non-digestable arabino-oligosaccharides (AOS). The prebiotic characteristics of selected carbohydrates in comparison to the established FOS were evaluated. Prebiotics are defined as ”selectively fermented ingredients that allow specific changes, both in the composition and/or activity of the gastrointestinal microbiota that confers benefits upon host well-being and health” [7]. Changes in the composition of the fecal microflora in connection to the size (chain length) of AOS were analyzed by qPCR, and the metabolic activity studies were focused on the metabolite production. The metabolic response to high-mass AOS was found to be the most similar to FOS, correlating to the microbial changes in the fecal microflora. High-mass AOS in comparison to the low-mass and base fraction caused the highest increase of metabolites putatively beneficial to the human GIT. However, an *in vitro* metabolic system altered by the presence of AOS, even with a full fecal microflora, might be developing differently than the one in the gut environment. As the bacterial composition differ greatly throughout the GIT [59], so can the metabolic response. Therefore, in order to evaluate the full response, just like in the probiotic studies, human clinical trials should be taken into consideration.

Probiotic and prebiotic influence on the host looks for the beneficial aspect of the bacterial flora. GIT is not only inhabited by beneficial microbiota, but also potential pathogens. A

balanced microflora requires that the bacterial composition work in a co-metabolic symbiotic relationship with the host, supporting the complex system. An unbalanced composition might potentially be the beginning of many diseases, such as inflammatory bowel diseases, in particular Ulcerative Colitis (UC) [205,206]. Several studies have shown that patients with UC have an altered bacterial microbiota [207-209]. Bacterial alterations might lead to the metabolic response of the microflora. Metabolomic footprinting has shown the significant differences in the metabolism between microflora from UC patients in relapse and remission or healthy individuals. In the UC studies Simulator of the Human Intestinal Microbial Ecosystem with incorporated mucin-covered microcosms (M-SHIME) was applied [210]. In search for the microbial metabolites exclusively, the dynamic gut model seems to be the best choice, possibly significantly lowering the impact of the individual diet as well as human-origin metabolites, still present in the fecal matter.

Throughout all of the studies in relation to the bacterial impact on the gut metabolome LCMS and DIMS in connection to the PCA was used. PCA in metabolomics is used extensively as an unsupervised method in search for the differences in metabolome between given settings [6,17]. However, a choice of chemical analysis in parallel with a sample preparation is always a matter of discussion. A wide range of analytical tools is used in metabolomics [18]. DIMS allows high-throughput analyses of biological samples. The major problem with DIMS technology is the matrix effect and even with the usage of TOF spectrometer, not good enough separation of metabolites. The mentioned effect can also compromise the sensitivity and accuracy of mass analysis [29]. Ion suppression caused by coeluting compounds and isobaric interferences are also a major disadvantage of DIMS. Liquid chromatography gives a good separation of metabolites. With LCMS a very wide range of metabolites can be covered by this method through the ionization in positive and negative mode with a very good sensitivity. LCMS sample preparation is very easy and straightforward. LCMS makes possible analysis of thermo-labile metabolites. A few drawbacks of this system are possible matrix effects, sometimes requiring the desalting of samples, limited structural information and identification, overcome by use of MS-MS techniques [34,35]. The choice of DIMS and LCMS in the study of complex metabolome was based on the positive aspects of both methods, our good results during the preliminary studies and instrument availability,

In conclusion, the experimental studies included in this thesis add to our understanding of bacterial impact on the gut metabolome. The new knowledge gained includes *Lactobacillus acidophilus* NCFM metabolite production and a possible alteration of mammalian host metabolome, AOS ability to change the overall composition of the fecal microbiota and their metabolic interactions with possible beneficial aspect of the high-mass carbohydrates. Additionally, a metabolomic-angled look on the UC allowed to constitute an important contribution to the understanding of the complex etiology of UC.

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