Top-down systems biology integration of conditional prebiotic modulated transgenomic interactions in a humanized microbiome mouse model

Francois-Pierre J Martin1,2,*, Yulan Wang1, Norbert Sprenger2, Ivan KS Yap1, Serge Rezzi2, Ziad Ramadan2, Emma Peré-Trepat2, Florence Rochat2, Christine Cherbut2, Peter van Bladeren2, Laurent B Fay2, Sunil Kochhar2, John C Lindon1, Elaine Holmes1 and Jeremy K Nicholson1,*

1 Division of Surgery, Oncology, Reproductive Biology and Anaesthetics, Department of Biomolecular Medicine, Faculty of Medicine, Imperial College London, London, UK and 2 Nestlé Research Center, Vers-chez-les-Blanc, Lausanne, Switzerland
* Corresponding authors. F-PJ Martin, Nestle´ Research Center, Vers-chez-les-Blanc, CH-1000 Lausanne 26, Switzerland. Tel.: + 41 21 785 8771; Fax: + 41 21 785 9486; E-mail: francois-pierre.martin@rdls.nestle.com or JK Nicholson, Department of Biomolecular Medicine, Imperial College London, Sir Alexander Fleming Building, South Kensington, London SW7 2AZ, UK. Tel.: + 44 20 7594 3195; Fax: + 44 20 7594 3226; E-mail: j.nicholson@imperial.ac.uk

Received 1.2.08; accepted 21.5.08

Gut microbiome–host metabolic interactions affect human health and can be modified by probiotic and prebiotic supplementation. Here, we have assessed the effects of consumption of a combination of probiotics (Lactobacillus paracasei or L. rhamnosus) and two galactosyl-oligosaccharide prebiotics on the symbiotic microbiome–mammalian supersystem using integrative metabolic profiling and modeling of multiple compartments in germ-free mice inoculated with a model of human baby microbiota. We have shown specific impacts of two prebiotics on the microbial populations of HBM mice when co-administered with two probiotics. We observed an increase in the populations of Bifidobacterium longum and B. breve, and a reduction in Clostridium perfringens, which were more marked when combining prebiotics with L. rhamnosus. In turn, these microbial effects were associated with modulation of a range of host metabolic pathways observed via changes in lipid profiles, gluconeogenesis, and amino-acid and methylamine metabolism associated to fermentation of carbohydrates by different bacterial strains. These results provide evidence for the potential use of prebiotics for beneficially modifying the gut microbial balance as well as host energy and lipid homeostasis.

Molecular Systems Biology 15 July 2008; doi:10.1038/msb.2008.40

Subject Categories: metabolic and regulatory networks; microbiology and pathogens

Keywords: galactosyl-oligosaccharides; human baby microbiota; Lactobacillus paracasei; Lactobacillus rhamnosus; metabolomics

This is an open-access article distributed under the terms of the Creative Commons Attribution Licence, which permits distribution and reproduction in any medium, provided the original author and source are credited. This licence does not permit commercial exploitation or the creation of derivative works without specific permission.

Introduction

Adult humans carry ca. 1.5 kg of gut microbial symbiotic and commensal organisms that are in intimate communication with the host metabolic and immune systems (Nicholson et al., 2005; Dethlefsen et al., 2007). This symbiosis is the result of a long period of co-evolution and co-adaptation between the host genotype and the complex and variable microbiome (Gill et al., 2006). Consequently, to be able to understand how the changes in environmental conditions and lifestyle influence human genetics and physiology, one needs to elucidate how these factors determine the distribution, activities and evolution of gut microbes, and subsequently transgenomic metabolic interactions (Xu et al., 2003; Backhed et al., 2005; Nicholson et al., 2005; Tannock, 2005; Sonnenburg et al., 2006; Blaut and Clavel, 2007; Turnbaugh et al., 2007). Thus, the gut microbiota can be regarded as an extra-genomic functional unit providing extra control mechanisms that affect the host’s nutritional status and health (Holmes and Nicholson, 2005; Nicholson et al., 2005; Bik et al., 2006; Martin et al., 2006; Eckburg and Relman, 2007). We have recently reported that exogenous gut microbiome components can be transplanted into a host and this results in modulation of the host calorific bioavailability via differential metabolism of bile acids, and we and others have surmised that related metabolic processes might be involved in common metabolic diseases such as obesity or type II diabetes (Dumas et al., 2006; Houten et al., 2006; Watanabe et al., 2006; Martin et al., 2007).

The effects of consuming live microbial supplements (probiotics) on the microbial ecology and on human health...
and nutritional status have been investigated extensively over many years (Collins and Gibson, 1999; Rastall, 2005; Sonnenburg et al., 2006; Martin et al., 2007b). It has been reported recently that probiotic consumption can lead to modification of the resident microbiota resulting in modulation of bile acid and lipid metabolism, and alter the recirculation and distribution of fat within the host organisms (Martin et al., 2008). Other reports suggest that the microbiota could be a contributing factor to obesity (Ley et al., 2006; Sonnenburg et al., 2006; Turnbaugh et al., 2006) and can, in addition, regulate host genes controlling lipid transport and deposition (Backhed et al., 2004).

As an alternative, the combined use of prebiotics and probiotics may have beneficial effects on health maintenance through modulating the microbial functional ecology (Collins and Gibson, 1999; Schrezenmeir and de Vrese, 2001). Prebiotics are non-digestible food ingredients, generally oligosaccharides, that modify the balance of the intestinal microbiota by stimulating the activity of beneficial bacteria, such as lactobacilli and bifidobacteria (Gibson and Roberfroid, 1995; Collins and Gibson, 1999). There is now considerable evidence that manipulation of the gut microbiota by prebiotics can beneficially influence the health of the host (Gibson and Roberfroid, 1995; Roberfroid, 1998; Delzenne and Kok, 2001; Sartor, 2004; Lim et al., 2005; Rastall, 2005; Parracho et al., 2007). In particular, many attempts have been made to control serum triacylglycerol concentrations through modification of dietary habits with regard to consumption of pre- and probiotics (Delzenne and Kok, 2001; Pereira and Gibson, 2002). Furthermore, unlike probiotics, prebiotics are not subject to biological viability problems and thus can be incorporated into a wide range of alimentary products (milk, yogurts, biscuits) and they target organisms that are natural residents of the gut microbiota (Gibson and Roberfroid, 1995). For example, oligosaccharides have been suggested to represent the most important prebiotic dietary factor in human milk, promoting the development of a beneficial intestinal microbiota (Kunz et al., 2000; Bode, 2006).

Nowadays, clinical trials support the claims of efficacy of pro- and prebiotic nutritional intervention with regard to various proposed beneficial health effects, and this has raised the requirement for providing additional evidence and for elucidation of the molecular bases of their action. This can be captured effectively only by studying the global system response of an organism to an intervention using top-down systems biology approaches. Metabolic profiling using high-resolution spectroscopic methods with subsequent multivariate statistical analyses is a well-established strategy for differential metabolic pathway profiling (Nicholson et al., 2005; Griffin and Nicholls, 2006; Ellis et al., 2007). Noticeably, the metabolic effects of various dietary modulations of gut microbiota have been successfully characterized using this approach (Wang et al., 2005, 2007; Martin et al., 2006; Stella et al., 2006; Goodacre, 2007; Rezzi et al., 2007). Recently, we have described that germ-free mice re-inoculated with a model of human baby microbiota (HBM mice) offer a simplified microbiome mouse model well adapted to assess the impact of nutritional intervention on the gut microbial functional ecosystem and subsequent effects on host metabolism (Martin et al., 2008). Interestingly, the microbiota model shows a number of similarities with the microbiota found in formula-fed neonates (Mackie et al., 1999). However, we also reported the limitations associated with gut colonization by a non-adapted microbiota and the subsequent alterations of host and microbial metabolism (Martin et al., 2007a).

The aim of the present study is to extend our previous investigations evaluating metabolic response to probiotics in HBM mice (Martin et al., 2008). In our previous study, we had shown alterations in carbohydrate and protein fermentation with subsequent effects on host lipid and energy metabolism, which were more marked with Lactobacillus paracasei than L. rhamnosus. In the current study, we compare the effects of consumption of a synthetic galactosyl-oligosaccharide (Pre1) with those due to consumption of an in-house preparation of galactosyl-oligosaccharides (Pre2). We have assessed the impact of prebiotics on the microbial balance and the mammalian metabolism of HBM mice supplemented with a probiotic, L. paracasei or L. rhamnosus (Figure 1). Here, we show a significant association of specific metabotypes obtained from urine, plasma, fecal extracts and intact liver tissue with changes of the gut microbiome induced by the prebiotic supplementation.

Results

Effects of pre- and probiotics on microbial composition and animal weight

The effects of prebiotics on the populations of microbiota in the jejunum and the feces are summarized in Table I. Fecal microbiota for the control groups (HBM alone, HBM + L. paracasei and HBM + L. rhamnosus) have previously been published (Martin et al., 2008). In the current and previous studies (Martin et al., 2008), the impact of probiotics with and without prebiotics on gut microbiota was assessed in the upper gut and the feces. The effects of probiotics are indeed expected along the whole gastrointestinal tract due to the great adaptability of lactobacilli to extreme aerobic/anaerobic conditions and low pH conditions (Tannock, 2004). However, most prebiotics are complex carbohydrates that escape digestion in the upper gastrointestinal tract and these are fermented by certain bacteria in the colon (Gibson and Roberfroid, 1995; Collins and Gibson, 1999). Nevertheless, the ability of galactosyl-oligosaccharides to modulate the upper gut microflora remains unclear and was thus also investigated. Our results provide evidence that the populations of microbiota in the fecal and jejunal content were modulated by prebiotic supplementation. In general, prebiotic supplementation slightly reduced the L. paracasei populations in both the fecal and jejunal content and increased fecal populations of Bifidobacterium breve and B. longum. Interestingly, supplementation with Pre2 was correlated with lower fecal populations of Clostridium perfringens in mice regardless of which probiotics they receive. The jejunal population of Bacteroides distasonis was decreased in HBM mice simultaneously supplemented with L. paracasei, whereas the number of fecal Escherichia coli was reduced in HBM mice simultaneously colonized with L. rhamnosus.

A three-component projection to latent structure discriminant analysis (PLS-DA) model of mean-centered microbial
counts in fecal and jejunal contents showed that the HBM control mice samples formed a distinct cluster (Figure 2A, black squares). Two subclusters of samples representing each of the probiotics administered either alone or in combination with prebiotics were observed in the plane described by Tcv1 and Tcv2 (red circles). These groups indicated that each probiotic exerted a systematic and unique effect on the microbial populations when colonized group than the other nutritional intervention groups and lower values when compared with other groups. The effects of the two prebiotics were compared with corresponding HBM + probiotics control mice, *, ** and *** designate significant difference at 95, 99 and 99.9% confidence level, respectively, —, probiotics not present in the gut microbiota.

Table 1 Microbial species counts in mouse fecal and jejunal contents

| Groups/log_{10} CFU | HBM (n = 10) | HBM + L. paracasei (n = 9) | HBM + L. paracasei + Pre1 (n = 9) | HBM + L. paracasei + Pre2 (n = 9) | HBM + L. rhamnosus (n = 9) | HBM + L. rhamnosus + Pre1 (n = 10) | HBM + L. rhamnosus + Pre2 (n = 9) |
|---------------------|-------------|---------------------------|-------------------------------|-------------------------------|---------------------------|---------------------------------|---------------------------------|
| **Feses**           |             |                           |                               |                               |                           |                                 |                                 |
| L. paracasei        | --          | 8.5 ± 0.2                 | 8.3 ± 0.3*                   | 8.1 ± 0.4**                   | --                       | --                              | --                              |
| L. rhamnosus        | --          | --                        | --                            | --                            | --                        | --                              | --                              |
| E. coli             | 9.2 ± 0.3   | 9.4 ± 0.3                 | 9.7 ± 0.3                    | 9.3 ± 0.2                     | 9.8 ± 0.5                 | 9.3 ± 0.2*                      | 9.3 ± 0.2*                      |
| B. breve            | 9.1 ± 0.2   | 7.8 ± 2.13                | 8.5 ± 1.5                    | 8.7 ± 1.5                     | 8.7 ± 0.3                 | 9.8 ± 0.3                       | 10 ± 0.4**                      |
| B. longum           | 8.2 ± 0.6   | 5.6 ± 1.9                 | 6.2 ± 1.6                    | 6.7 ± 1.8                     | 6.3 ± 0.5                 | 7.7 ± 1.2**                     | 9.3 ± 1.04**                    |
| S. aureus           | 7.4 ± 0.3   | 6.3 ± 0.3                 | 6.3 ± 0.5                    | 6.1 ± 0.7                     | 6.6 ± 0.5                 | 6.1 ± 0.4                      | 6.4 ± 0.9                      |
| S. epidermidis      | 4.8 ± 0.4   | 4.9 ± 1.2                 | 4.5 ± 0.9                    | 3.8 ± 0.4                     | 4.0 ± 0.5                 | 3.7 ± 0.7                      | 6.0 ± 1.5                      |
| C. perfringens      | 7.2 ± 0.3   | 7.0 ± 0.5                 | 6.5 ± 1.0                    | 5.9 ± 0.6**                   | 5.7 ± 1.0                 | 6.6 ± 1.1                      | <5.0                            |
| Bacteroides distasonis | 10.3 ± 0.2 | 10.4 ± 0.2                | 10.1 ± 0.6                   | 10.1 ± 0.4                    | 10.1 ± 0.4                | 10.2 ± 0.3                      | 10.3 ± 0.3                     |

**Jejunum**

| L. paracasei        | --          | 4.2 ± 1.8                 | 2.9 ± 0.8                    | 2.6 ± 0.9                     | --                        | --                              | --                              |
| L. rhamnosus        | --          | --                        | --                            | 3.6 ± 1.3                     | 3.1 ± 1.3                 | 3.0 ± 0.8                      |
| E. coli             | 3.3 ± 1.3   | 5.1 ± 1.9                 | 4.0 ± 0.8                    | 3.7 ± 1.2                     | 5.2 ± 1.9                 | 4.2 ± 1.3                      | 4.4 ± 0.8                      |
| B. breve            | 2.7 ± 1.4   | 2.5 ± 1.0                 | 2.7 ± 1.3                    | 3.0 ± 1.4                     | 2.4 ± 0.8                 | 4.0 ± 1.6**                    | 4.0 ± 0.9**                    |
| B. longum           | <2.0        | <2.0                      | <2.0                          | <2.0                          | <2.0                      | <2.0                           | <2.0                           |
| S. aureus           | 4.1 ± 0.9   | 3.8 ± 1.3                 | 4.1 ± 0.9                    | 3.7 ± 0.7                     | 4.2 ± 0.7                 | 3.0 ± 1.3*                     | 4.0 ± 0.5                      |
| S. epidermidis      | <3.0        | <3.0                      | <3.0                          | <3.0                          | <3.0                      | <3.0                           | =3.0                           |
| C. perfringens      | 3.4 ± 1.1   | 4.5 ± 1.2                 | 3.4 ± 0.7                    | 2.9 ± 1.1                     | 4.7 ± 1.3                 | 3.4 ± 1.0*                     | 3.2 ± 0.5*                     |
| Bacteroides distasonis | 3.4 ± 1.6 | 4.8 ± 1.6                 | 3.2 ± 1.2*                   | 3.3 ± 0.9                     | 3.8 ± 1.7                 | 4.0 ± 1.3                      | 4.1 ± 1.3                      |

Key: Log_{10}CFU (colony forming unit) given per gram of wet weight of feces or wet weight of jejunal content. Data are presented as mean ± s.d. The average values obtained from the HBM + probiotics mice supplemented with prebiotics were compared with corresponding HBM + probiotics control mice, *, ** and *** designate significant difference at 95, 99 and 99.9% confidence level, respectively, —, probiotics not present in the gut microbiota.
superimposed on the probiotic background were further differentiated along component Tcv3. Multivariate data analysis highlighted that prebiotic intervention was correlated with increased *B. longum* and *B. breve*, and lower numbers of *E. coli* and *C. perfringens*.

No effect of prebiotic supplementation on animal body weight was observed (Supplementary Table 1).

**Quantification of short-chain fatty acids in the cecum**

Several short-chain fatty acids (SCFAs), namely acetate, propionate, isobutyrate, n-butyrate and isovalerate, were identified and quantified from the cecal content using gas chromatography (GC) with flame ionization detection. The results, presented in Table II, are given in μmol per gram of dry cecal material and as mean ± s.d. for each group of mice. The data for the control groups (HBM colonized mice without further intervention and HBM colonized mice after administration of a probiotic) have previously been published, but are included here for comparative purposes (Martin *et al.*, 2008). The effect of prebiotic treatment on the production of SCFAs was limited to a reduction in the production of propionate and butyrate in HBM mice receiving *L. rhamnosus* combined with Pre2 and a reduction in isobutyrate in HBM mice receiving *L. paracasei* combined with Pre2 (Table II). Although cecal L- and D-lactate were not actually measured in the present

---

**Figure 2** PLS-DA scores plots (A–C) and loading plots for the three predictive components (D, E) derived from PLS-DA model of log10 CFU (colony forming unit) for the different bacterial species measured for fecal and jejunal samples from HBM control (black square), HBM + *L. rhamnosus* (red dot), HBM + *L. rhamnosus* + Pre1 (blue diamond), HBM + *L. rhamnosus* + Pre2 (green triangle), HBM + *L. rhamnosus* (red circle), HBM + *L. rhamnosus* + Pre1 (purple open diamond) and HBM + *L. rhamnosus* + Pre2 (green open triangle). Loadings represent the bacterial populations, beginning with J or F for jejunal or fecal counts, respectively. The model has been calculated with four predictive components and mean-centered data, $R^2 = 76.5\%$, $Q^2 = 51.3\%$. Key: B.a., *Bacteroides distasonis*; B.b., *Bifidobacterium breve*; B.l., *Bifidobacterium longum*; C.p., *Clostridium perfringens*; E.c., *Escherichia coli*; L.p., *Lactobacillus paracasei*; L.r., *Lactobacillus rhamnosus*; S.a., *Staphylococcus aureus*; S.e., *Staphylococcus epidermidis*. 

---

Prebiotics modulation of mammalian metabolism

F-PJ Martin *et al*
Table II SCFA content in the cecum from the different groups

| SCFA concentration | Acetate | Propionate | Isobutyrate | Butyrate | Isovalerate |
|---------------------|---------|------------|-------------|----------|-------------|
| HBM + L. paracasei (n = 7) | 59.7 ± 11.4 (65.4 ± 4.4) | 25.3 ± 6.6 (27.7 ± 3.9) | 1.4 ± 0.5 (1.6 ± 0.4) | 1.7 ± 0.5 (1.9 ± 0.3) | 3.1 ± 0.5 (3.5 ± 0.7) |
| HBM + L. paracasei + Pre1 (n = 9) | 73.4 ± 25.0 (70.1 ± 4.9)* | 23.9 ± 5.3 (23.6 ± 3.6) | 1.1 ± 0.2 (1.1 ± 0.2)** | 2.3 ± 0.5 (2.3 ± 0.6) | 2.8 ± 0.4 (2.9 ± 0.7) |
| HBM + L. paracasei + Pre2 (n = 9) | 80.4 ± 4.2 (72.3 ± 5.7)* | 22 ± 3.4 (21.8 ± 4) | 1.1 ± 0.1* (1.1 ± 0.4)* | 1.6 ± 0.9 (1.6 ± 0.7) | 2.9 ± 0.2 (3.1 ± 1.2) |
| HBM + L. rhamnosus (n = 9) | 40.6 ± 8.0 (61.3 ± 4.1) | 20.3 ± 2.8 (31 ± 3.7) | 0.8 ± 0.2 (1.3 ± 0.3) | 2.1 ± 0.4 (3.3 ± 0.5) | 2.1 ± 0.5 (3.2 ± 0.5) |
| HBM + L. rhamnosus + Pre1 (n = 10) | 54.7 ± 22.7 (68.2 ± 5.2)** | 19 ± 4 ± 1 (24.9 ± 4.1)*** | 0.9 ± 0.2 (1.2 ± 0.3) | 1.9 ± 0.4 (2.5 ± 0.6)*** | 2.3 ± 0.4 (3.2 ± 0.9) |
| HBM + L. rhamnosus + Pre2 (n = 9) | 45.3 ± 19.5 (69.8 ± 6)* | 14.7 ± 4.4* (24.1 ± 4.9)* | 0.8 ± 0.3 (1.3 ± 0.4) | 0.9 ± 0.3 ± 1.5 ± 0.4)*** | 2.0 ± 0.8 (3.3 ± 1.1) |

Keys: Data are presented as mmol per gram of dry cecal content and as means ± s.d. The relative composition of SCFAs in the total content is given in percentage in parentheses. The average values obtained from the HBM + probiotics mice supplemented with prebiotics were compared with corresponding HBM + probiotics alone mice; *, ** and *** designate significant difference at 95, 99 and 99.9% confidence level, respectively.

**Fecal metabolic profiles**

Pre1 and Pre2 caused marked effects on the metabolic profiles of fecal extracts of mice colonized with HBM and L. paracasei, and these effects included a marked increase in the concentrations of some as yet unassigned SCFAs and a decreased level of metabolites characteristic of cecal content of oligosaccharide (O1, O3), which were associated with a reduced levels of metabolites characteristic of cecal content of oligosaccharide (O1, O3), which were associated with a reduced levels of metabolites characteristic of cecal content of oligosaccharide (O1, O3). Pre2 treatment was also correlated with increases in the other unassigned oligosaccharides (O2, O4, and O5). O-PLS-DA models of a pairwise comparison between Pre1 and Pre2 showed the differences in the content of oligosaccharides O1 and O3 between the groups of HBM and L. paracasei supplemented with Pre1. Pre2-induced metabolic changes of the cecal content of mice colonized with HBM and L. paracasei are further illustrated in Figures 3 and 4. The changes in the content of oligosaccharides O1 and O3 may result from the digestion of prebiotics by the gut microbiota.
In HBM mice supplemented with *L. paracasei*, unique effects of Pre1 included elevated levels of arginine and citrulline, and reduced octanoic acid (caprylate) in the fecal composition, whereas unique features of Pre2 ingestion included a decrease in the levels of glucose, lysine, butyrate, isovalerate and propionate. When combined with *L. paracasei* supplementation, both prebiotic treatments were associated with a reduction in the content of lactose. In addition, in the feces of HBM mice supplemented with *L. rhamnosus*, Pre1 induced higher levels of arginine and citrulline, whereas Pre2 caused a decrease in lysine, butyrate and isovalerate. In HBM mice supplemented with *L. rhamnosus*, both prebiotic treatments were associated with a reduction in the content of lactose, glucose, glutamate and octanoate.

Liver metabolite profiles
The liver of mice colonized with *L. paracasei* and receiving either of the prebiotics was metabolically differentiated from those fed with probiotics alone, as indicated by the increased levels of glycogen, trimethylamine (TMA), polyunsaturated fatty acids (PUFAs) and a range of amino acids (i.e. leucine, isoleucine, glutamine, glutamate, glycine) and a decreased concentration of triglycerides (Supplementary Figure 2A and B and Table III). Moreover, Pre2 induced specific increases in the levels of trimethylamine-N-oxide (TMAO) in *L. paracasei* colonized animals.

In addition, *L. rhamnosus* colonized HBM mice supplemented with Pre1 were characterized by increased levels of amino acids and PUFAs. Supplementation with Pre2 was specifically associated with a reduction in the level of glycogen and an increase in TMAO and phosphatidylcholine.

Urinary metabolite profiles
Prebiotic administration also affected the urinary metabolic profiles of mice colonized with *L. paracasei*. These changes were mainly manifested in decreased concentrations of a putative mixture of lipids (unidentified lipids (ULp), chemical shifts: 0.89(m), 1.27(m), 1.56(m), 2.25(m)) and an increase in 1-methylnicotinamide in mice fed with Pre1 or Pre2. In addition, consumption of prebiotics was correlated with higher levels of an unknown compound U1 (1H NMR chemical shift: 3.80(m), 4.30(t) as given by statistical total correlation spectroscopy (STOCSY) analysis; Cloarec et al., 2005a) in urine. Pre1 also caused decreased concentrations of phenylacetyl-glycine, N-acetyl- and O-acetyl-glycoproteins, and tryptamine and increased levels of citrate. Animals supplemented with Pre2 showed elevation in the levels of glycerate, creatine and TMA, which was associated with a reduction of α-keto-isovalerate, arginine and citrulline. In addition, consumption of Pre2 was correlated with higher levels of U1 and another unknown compound U2 (1H NMR chemical shifts: 3.80(m), 4.30(t)) of creatinine.

In contrast, *L. rhamnosus* colonized mice treated with both prebiotics showed higher urinary excretion of creatine, taurine and U1, and a reduction in urinary levels of arginine and citrulline. Feeding HBM mice with *L. rhamnosus* and Pre2 led to increased urinary concentrations of ULp, TMA and U2, and decreased levels of α-keto-isovalerate and creatinine.

Correlation analysis of inter-compartment metabolite functional relationships
As the major changes following intervention with combined use of pre- and probiotics occurred in the fecal and liver matrices, a correlation analysis was conducted to identify any...
| Metabolite | Chemical shift and multiplicity | HBM + L. r. Pre1 | HBM + L. r. Pre2 | HBM + L. r. Pre3 | HBM + L. r. Pre4 | HBM + L. r. Pre5 |
|------------|-------------------------------|------------------|------------------|------------------|------------------|------------------|
| Glu        | 2.34 (m)                      | 0.4±0.1          | 0.8±0.4          | 0.6±0.2          | 0.4±0.1          | 0.5±0.3          |
| Gln        | 2.44 (m)                      | 1.3±0.3          | 1.8±0.4          | 1.8±0.4          | 1.9±0.5          | 1.9±0.6          |
| TGL        | 1.27 (s)                      | 0.2±0.1          | 0.5±0.2          | 0.4±0.1          | 0.4±0.1          | 0.4±0.1          |
| PUFAs      | 5.26 (m)                      | 0.2±0.1          | 0.4±0.1          | 0.2±0.1          | 0.2±0.1          | 0.2±0.1          |
| Glycogen   | 5.45 (m)                      | 0.4±0.1          | 0.6±0.2          | 0.5±0.1          | 0.5±0.1          | 0.5±0.1          |
| Oligosaccharides O1 | 3.94 (m) | 0.2±0.1          | 0.4±0.1          | 0.4±0.1          | 0.4±0.1          | 0.4±0.1          |
| Oligosaccharides O2 | 5.43 (m) | 0.2±0.1          | 0.4±0.1          | 0.4±0.1          | 0.4±0.1          | 0.4±0.1          |
| Oligosaccharides O3 | 3.67 (m) | 0.2±0.1          | 0.4±0.1          | 0.4±0.1          | 0.4±0.1          | 0.4±0.1          |

**Table III: Summary of influential metabolites for discriminating H-NMR spectra of liver, fecal extracts and urine**

Data are presented as area-normalized intensities (10^11 a.u.) of representative metabolite signals expressed as means ± s.d. The values for the HBM mice supplemented with probiotics in combination with prebiotics were compared with HBM control mice fed with the probiotics alone. a, * and ** designate significant difference at 90, 95, 99 and 99.9% confidence level, respectively.
latent metabolic links between these two biological compartments (Figure 5). Such analyses have been carried out on groups of animals that received the same probiotic combined with prebiotics or not. Pixel maps obtained from the two groups of animals showed different intra- and inter-compartment correlation patterns, which highlighted the metabolic
Figure 5 Integration of inter-compartment metabolic correlations. The pixel maps were derived from correlations between liver and fecal metabolites found to be significantly different with nutritional intervention in each group of mice colonized with one type of probiotic. The intra- and inter-compartmental metabolite correlations are displayed for HBM mice supplemented with L. paracasei probiotics down the diagonal from top-left to bottom-right, and with L. rhamnosus probiotics up the diagonal from top-left to bottom-right. The cutoff value of 0.4 was applied to the absolute value of the coefficient |r| of the test statistic. Correlation values are displayed as a color-coded pixel map according to correlation value (gradient of red colors for positive values and gradient of blue colors for negative values). Key: Ala, alanine; Arg, arginine; Gln, glutamine; Glu, glutamate; Gly, glycine; Ileu, isoleucine; Lys, lysine; PC, phosphocholine; PUFA, polyunsaturated fatty acid; TGL, triglycerides; TMA, trimethylamine; TMAO, trimethylamine-N-oxide.

Correlation analysis of microbiotal variation and SCFAs

A correlation analysis was applied to investigate the connections between levels of fecal and jejunal microbiota and the cecal SCFAs using bipartite graphical modeling (Figure 6). Positive and negative correlations between nodes show the multicolinearity between SCFAs and gut bacteria, whose concentrations are interdependent such as in the case of substrate–product biochemical reactions. Correlation analysis derived from SCFAs and fecal/jejunal microbiota profiles offered a unique approach to describe intra-group sources of variability and subtle alterations in SCFAs in relation to gut bacterial changes. By comparing the networks obtained with different treatments, we can highlight significant differential patterns, suggesting different functional ecology in relation to different microbial populations and activities. HBM mice supplemented with different probiotic/prebiotic combinations show remarkably different SCFA/microbial correlation networks (Figure 6), indicating that probiotic and prebiotic modulation of the microbiome can result in specific functional ecological changes. In particular, we observed that microbial changes in the upper gut and fecal pellet showed a functional relationship with the intestinal content of SCFAs. Such data can help to generate testable hypotheses on differential bacterial metabolism in response to a stressor.

In particular, network analysis for HBM mice supplemented with L. paracasei revealed that dietary oligosaccharide supplementation induced significant changes in the functional linkage between the acetate and propionate levels, and lactobacilli, bifidobacteria, Bacteroides distasonis and...
C. perfringens. Interestingly, fecal bacterial changes showed strong correlations with the cecal composition of SCFAs in mice not supplemented with prebiotics (Figure 6A). Animals receiving prebiotics showed a greater number of statistically significant correlations between the jejunal microbiota changes and the SCFAs. Moreover, Pre1 induced negative
and positive correlations between SCFAs and \textit{C. perfringens} and \textit{B. breve}, respectively (Figure 6B). Pre2 supplementation was associated with positive correlations between SCFAs (acetate and propionate) and both \textit{Bacteroides distasonis} and \textit{B. breve} (Figure 6C). In contrast, negative correlations were observed for \textit{S. aureus} with most of the SCFAs (Figure 6C).

When HBM mice received \textit{L. rhamnosus} with or without prebiotics, the microbiome/SCFAs network showed a simpler structure. Such observations suggest different bacterial interactions in both HBM + prebiotic models. Available microbial data support this idea as seen, for instance, with the specific inhibition of \textit{B. breve} growth with \textit{L. paracasei} but not with \textit{L. rhamnosus}. In contrast with animals supplemented with \textit{L. paracasei} alone, variation in the intestinal bacterial populations from mice that received \textit{L. rhamnosus} was strongly correlated with the cecal composition in SCFAs (Figure 6D). Moreover, Pre1 induced correlations between the cecal content in acetate and the balance between \textit{B. breve}, \textit{B. longum} and \textit{C. perfringens}. Interestingly, Pre2 appeared to initiate functional relationships between the main SCFAs (acetate, propionate and butyrate) and \textit{Bacteroides distasonis}, \textit{C. perfringens} and lactobacilli.

\textbf{Discussion}

We have shown specific effects of two prebiotics on the microbial populations of HBM mice when co-administered with two probiotics. These microbial changes were associated with specific host metabolic phenotypes, for example, variations in the fecal carbohydrate content, reduction in the levels of hepatic triglyceride content and increased hepatic concentrations of PUFAs and hepatic gluconic amino acids. These data provide further evidence for the critical involvement of prebiotics in host metabolism through modulation of the gut microbiome.

\textbf{Effects of prebiotics on gut functional ecology}

Previous work on humans has described the fragile equilibrium between the host and beneficial gut bacteria (lactobacilli, bifidobacteria) and potentially detrimental species (\textit{Clostridium} spp, \textit{Staphylococcus} spp and the members of the Enterobacteria and \textit{Bacteroides} groups), and this ultimately determines the health and nutritional status of the host (Collins and Gibson, 1999; Pereira and Gibson, 2002; Gopal et al, 2003; Martin et al, 2006; Sonnenburg et al, 2006). Our data provide additional evidence that the populations of beneficial bacteria in the gastrointestinal tract can, to some extent, be controlled with dietary interventions, here based on supplementation with galactosyl-oligosaccharides. Here, increases in the fecal populations of beneficial bacteria, namely \textit{B. longum} and \textit{B. breve} (Table I), were specifically associated with supplementation of prebiotics, Pre2 offering a greater ability to modulate the gut microbiota in HBM mice compared with Pre1. Galacto-oligosaccharides are the preferred growth substrates for bifidobacteria and lactobacilli that have an extraordinary ability to acquire and degrade oligosaccharides (Kikuchi et al, 1992; Ito et al, 1993; Rycroft et al, 2001; Tzortzis et al, 2005; Macfarlane et al, 2006). Pure bacterial cultures indicated that \textit{L. rhamnosus} and \textit{L. paracasei} were not able to grow on culture media containing Pre1, whereas \textit{B. breve} and \textit{B. longum} did not show any difference in growth when cultured on Pre1, glucose or lactose media (unpublished data).

In addition, the ability of \textit{B. longum} (Hopkins et al, 1998; Macfarlane et al, 2008) and \textit{B. breve} (Djouzi et al, 1995) to utilize extensively galacto-oligosaccharides for growth was previously reported \textit{in vivo}, whereas only a few \textit{Lactobacillus} species could use this substrate efficiently, unlike \textit{L. rhamnosus} (Gopal et al, 2001). This bacterial capacity is strongly dependent on the pattern of glycosidic linkages present in the galacto-oligosaccharides and thus on the existence of specific \(\beta\)-galactosidases, as evidenced for \textit{B. longum} by genome analysis (Schell et al, 2002).

It has been previously reported that bacterial fermentation of carbohydrate may result in inhibition of the growth of pathogens by acidification of the environment through the production of large quantities of carboxylic acids (Kikuchi et al, 1992; Ito et al, 1993; Rowland, 1993; Gibson and Roberfroid, 1995; Djouzi and Andrieux, 1997). In the current study, prebiotic supplementation resulted in a reduction in \textit{E. coli} and \textit{C. perfringens} bacterial counts in the feces (Table I), which is in agreement with previous reports (Rycroft et al, 2001; Tzortzis et al, 2005). Our results suggest that the association of Pre2 with \textit{L. rhamnosus} enables a more significant reduction in pathogenic \textit{C. perfringens} and an increase in health-promoting \textit{B. longum} populations in feces and jejunal content. However, measures of cecal SCFAs did not reveal significant alterations in total concentrations and composition with prebiotic treatment (Table II), whereas changes were more marked in the stool (Table III). Therefore, it remains unclear if the gut microbial changes result directly from the fermentation of galactosyl-oligosaccharides and acidification of the luminal environment. Moreover, the significant increase in fecal bifidobacteria suggests that a different bacterial fermentation may occur in the colon, and measures of cecal pH and of colonic content of SCFAs will help in the interpretation in future studies.

However, our results show that the type of prebiotic (Pre1 or Pre2) entering the large intestine has differential effects on bacterial metabolism and is in agreement with the different abilities of bacteria such as lactobacilli, bifidobacteria and \textit{Bacteroides} spp to hydrolyze carbohydrates, as reported previously (Hidaka and Hirayama, 1991; Djouzi and Andrieux, 1997). Application of network analysis to display the relationships between cecal SCFAs and microbial profiles revealed different intra-group patterns (Figure 6). These observations suggest that prebiotics induced a specific functional ecology in relation to different microbial populations and activities. For instance, the negative correlations between SCFAs and \textit{C. perfringens} and \textit{E. coli}, and positive correlations between SCFAs and bifidobacteria, lactobacilli and \textit{Bacteroides distasonis} consistently indicate a link between SCFA production and certain changes in bacterial populations, such as reduction in pathogens. Moreover, \textit{C. perfringens} being a primary butyrate producer, the specific anti-correlation with butyrate may also indicate that in response to stressors, \textit{C. perfringens} activities may be shifted and stimulated. In particular, measures of bacterial activities in similar mouse models showed that the reduction of \textit{C. perfringens} counts was associated with an increased activity in response to Pre2 supplementation (unpublished data).
In HBM mice supplemented with _L. paracasei_, populations of lactobacilli were slightly reduced with both prebiotic supplementations, and bifidobacteria showed only upward trends, which suggested a competition for the prebiotics between bifidobacteria and _L. paracasei_. Moreover, the observation of higher fecal content of oligosaccharides O1 and O3 specific to HBM mice fed with prebiotics and _L. paracasei_ indicated that the microbiota may use these substrates poorly when compared with groups with _L. rhamnosus_. This information suggests a higher efficiency of bacterial hydrolysis and intestinal absorption of dietary oligosaccharides in animals fed with prebiotics and _L. rhamnosus_ (Table III and Supplementary Figures 1 and 3).

Furthermore, ingestion of galacto-oligosaccharides or fructo-oligosaccharides is known to specifically induce bacterial hydrolysis of the substrate (Djouzi and Andrieux, 1997), as well as to modulate some bacterial activities, including glycolytic properties, hydrolysis of oligosaccharides and glucuronides, reduction of nitro-compounds, and formation of phenols and indoles (Mitsuoka et al., 1987; Ito et al., 1993; Rowland, 1993). In the current study, decreased levels of lysine in feces (Table III), isobutyrate in cecum (Table II) and N-acetyl-glycoproteins in the urine (Table III) suggest that prebiotic treatment decreased overall bacterial proteolysis in animals also receiving _L. paracasei_ (Macfarlane et al., 1992; Hallson et al., 1997; Metges, 2000). Investigation of the metabolite changes in urinary excretion showed a significant decrease in the concentrations of microbial co-metabolites PAG and tryptamine (Goodwin et al., 1994; Smith and Macfarlane, 1996), which also supports decreased bacterial proteolytic activities. Altogether, our data suggest that prebiotics intervention may reduce proteolytic activities previously ascribed to the basal metabolism of _L. paracasei_ on casein medium (Martin et al., 2008), which is in agreement with the reduced number of these bacteria observed in this study.

In addition, methylamines, as a class of compounds, are another well-documented example of metabolites derived from host–microbial interactions produced within the large intestine (Smith et al., 1994). A significant fraction of ingested choline is converted by microbial enzymes to TMAO and tMAO (Zeisel et al., 1983), which is either oxidized to TMAO in the liver or excreted into the urine (Smith et al., 1994). Increases in the levels of TMA and TMAO in the liver and TMA in the urine indicate that changes in methylamine metabolism were induced by prebiotics, the changes being more marked with Pre2 supplementation (Table III).

**Impact of prebiotics on host energy and lipid homeostasis**

In parallel to gut microbial changes, relative reduction of hepatic triglycerides and increased concentrations of PUFAs were observed in mice supplemented with prebiotics. Non-digestible but fermentable carbohydrates were reported to decrease triglycerides in both serum and liver via modulation of the activity and gene expression of the lipogenic enzymes (Delzenne and Kok, 1998, 2001; Roberfroid and Delzenne, 1998; Pereira and Gibson, 2002). For instance, fatty acid synthase is sensitive to nutrients and hormones (Girard et al., 1997), whereas insulin and glucose are essential factors regulating fatty acid and triglyceride synthesis (Katsurada et al., 1990; Girard et al., 1997). Moreover, fructan-type prebiotic feeding may reduce the ability of isolated hepatocytes to synthesize and secrete triglycerides by 54% (Kok et al., 1996), as well as their ability to esterify fatty acids into triacylglycerols (Fiordaliso et al., 1995). A similar mechanism might exist with galactosyl-oligosaccharides, which may explain the relative increase in the NMR signals of PUFA-containing phospholipids in the current study. PUFAs can act by directing fatty acids away from triglyceride storage and toward oxidation, and can also enhance glucose flux to glycolgen (Kliwer et al., 1997). These processes are supported by the higher content of hepatic glutamine and branched-chain amino acids observed here, which would have a lower contribution to the citric acid cycle. No significant effects of prebiotics on the plasma metabolic profiles were observed here (Table III). Previously, we described that single probiotic supplementation reduced the levels of plasma lipoproteins in HBM mice by modulating the absorption of the dietary long-chain PUFAs (Martin et al., 2008). Here, we report that similar blood plasma metabolic profiles, associated with reduction of the triglyceride content in the liver, can be obtained when combining the probiotic with prebiotic supplements (Table III).

Notably, animals fed with _L. paracasei_ in combination with prebiotics showed the most significant hepatic reduction in triglycerides, which was associated with a high fecal content of oligosaccharides. Previous studies showed that some prebiotics induce changes in lipogenic enzyme activities by reducing postprandial insulinemia and glyceremia (Kok et al., 1998; Delzenne and Kok, 2001) through stimulation of the intestinal release of hormonal mediators (Morgan, 1996), or through modification of the intestinal absorption of carbohydrates (Stanley and Newsholme, 1985) and shortening small intestinal transit time (Roberfroid and Delzenne, 1998). Our results suggest that a similar mechanism may be involved in mice supplemented with prebiotics co-administered with _L. paracasei_, as the higher concentrations of fecal oligosaccharides may reflect poorer digestion and absorption resulting in lower energy generation from carbohydrates, with a consequent switch to fat metabolism. However, further work is needed to understand the functional link between the residual fecal carbohydrate and the digestion of prebiotics by the gut microbiota, for instance by assessing experimentally the metabolic abilities of the bacterial species to utilize the prebiotics. Moreover, recent studies showed that the microbial processing of dietary oligosaccharides modifies monosaccharide uptake from the gut by regulating the activity of host monosaccharide transporters, which can result in various changes in hepatic metabolism, including modulation of synthesis and deposition of triglycerides in adipocytes and increased glycogenesis (Backhed et al., 2004). Here, prebiotic supplementation was associated with relatively increased levels of glutamate, glutamine, branched-chain amino acids and alanine in the liver, as well as hepatic glycogen accumulation when mice were specifically fed with _L. paracasei_, which suggests stimulated gluconeogenesis and glycogenesis (Table III).

The animals that received _L. rhamnosus_ in combination with either prebiotics showed elevated levels of urinary...
taurine and creatine, which is likely to be related to higher muscular activity due to supplementation of the feed with new sources of carbohydrates (Cuisinier et al., 2001). Moreover, it has been shown that adipocyte-derived hormones, whose expression correlates with adipocyte lipid content, can increase energy expenditure in mice (Backhed et al., 2004). These changes suggest that HBM supplemented with *L. rhamnosus* supplies the host metabolism with new sources of carbohydrates more efficiently, which leads to changes in energy expenditure.

In conclusion, integrative systemic metabolic and microbiome profiling demonstrated the importance of nutritional intervention based on prebiotics and probiotic combinations in determining the host metabolic status and the levels of a diverse range of compounds in multiple pathways. Our data highlight that prebiotic nutritional intervention is a key factor in determining the resulting host metabolic phenotypes. The perspective of inducing unique changes in the host metabolism triggered by unique combinations of prebiotics and probiotics establishes an important step forward in the efforts to develop tailored nutritional solutions at an individual level.

**Materials and methods**

**Animal handling procedure and supplementation of probiotics and prebiotics**

All animal studies were carried out under appropriate national guidelines at the Nestlé Research Center (Lausanne, Switzerland). The model of HBM consists of seven bacterial strains that were isolated, using a previously described method (Guigoz et al., 2002), from the stool of a 20-day-old female baby who was given birth by Caesarean section (group A, n=9) or *L. rhamnosus* (group B, n=9; group C, n=9) or *L. rhamnosus* (group E, n=10; group F, n=9). With an average consumption of 5 ml of drinking water per mouse per day, each animal received 10⁶ CFU probiotics per day. Fecal pellets and morning spot urine samples were collected and frozen for NMR spectroscopy at the end of the 2 weeks of nutritional intervention. An additional fecal pellet was also collected in sterile condition for microbial profiling. Urine samples were not obtained for every animal, as some mice had an empty bladder at the time of termination (i.e. total urine samples per group was A, n=6; B, n=8; C, n=6; D, n=8; E, n=6; F, n=7). Animals were weighed and then euthanized. Blood (400 μl) was collected into Li-heparin tubes and the plasma was obtained after centrifugation and then frozen at -80 °C. All animal studies were carried out under appropriate national guidelines at the Nestlé Research Center (Lausanne, Switzerland). All animal studies were carried out under appropriate national guidelines at the Nestlé Research Center (Lausanne, Switzerland).

A total of 65 C3H female germ-free mice, aged 6 weeks, received a single dose of HBM bacterial mixture and were fed with a standard semisynthetic germ-free rodent diet for 2 weeks, as described previously, to allow establishment of the HBM (Martin et al., 2008). The full trial design is given in Figure 1. A control group of HBM mice (n=10) received a saline drink containing Man, Rogosa and Sharpe (MRS) culture medium and were fed with a basal diet containing 2.5% of a glucose–lactose mix (1.25% each) for 2 additional weeks. Two groups of HBM mice were given daily a probiotic supplement, either *L. paracasei* (group A, n=9) or *L. rhamnosus* (group D, n=9), containing 10⁶ probiotic bacteria in MRS per day mixed with saline solution and were also fed with the basal diet. Two groups of HBM mice were fed with a diet containing 3 g per 100 g diet of commercially available galactosyl-oligosaccharide prebiotics (Vivinal-GOS, Borculo Domo Ingredients, The Netherlands), called Pre2 here. Pre2 is composed of 80% of Pre1 and 20% of a mixture containing additional galactosyl-oligosaccharide structures, the latter being primarily composed of DP 3 oligomers with β-1,3 and β-1,6 linkages. The control diet was supplemented with lactose and glucose to control for the lactose and glucose that were added to the experimental diets by means of galactosyl-oligosaccharide preparations. Additionally, all groups received daily a fresh pellet either *L. paracasei* (group B, n=9; group C, n=9) or *L. rhamnosus* (group E, n=10; group F, n=9). With an average consumption of 5 ml of drinking water per mouse per day, each animal received 10⁶ CFU probiotics per day. Fecal pellets and morning spot urine samples were collected and frozen for NMR spectroscopy at the end of the 2 weeks of nutritional intervention. An additional fecal pellet was also collected in sterile condition for microbial profiling. Urine samples were not obtained for every animal, as some mice had an empty bladder at the time of termination (i.e. total urine samples per group was A, n=6; B, n=8; C, n=6; D, n=8; E, n=6; F, n=7). Animals were weighed and then euthanized. Blood (400 μl) was collected into Li-heparin tubes and the plasma was obtained after centrifugation and then frozen at -80 °C. All animal studies were carried out under appropriate national guidelines at the Nestlé Research Center (Lausanne, Switzerland).

**Microbial profiling of fecal and jejunal contents**

Briefly, for each mouse, a fecal pellet was homogenized in 0.5 ml Ringer solution supplemented with 0.05% (w/v) L-cysteine (HCl). For fecal and jejunal samples, solutions at different dilutions were plated on selective and semiselective culture media to assess the bacterial populations, *B. breve* and *B. longum* on Eugon Tomato medium (Chemie Brunschwig, Switzerland), *L. paracaset* and *L. rhamnosus* on MRS medium (Chemie Brunschwig) with antibiotics (phosphomycin, sulfamethoxazole and trimethoprim) medium (Sigma, Switzerland), *C. perfringens* on NN-agar medium (Chemie Brunschwig), *E. coli* on Chapman medium (BioMérieux, Switzerland) and *S. aureus* on Chapman medium (BioMérieux). The bacterial cultures of *E. coli*, *S. aureus* and *S. epidermidis* were incubated at 37 °C under aerobic conditions for 24 h and those of *B. longum*, *B. breve*, *L. rhamnosus*, *L. paracaset*, *Bacteroides distasonis* and *C. perfringens* under anaerobic conditions for 48 h.

**Gas chromatographic analysis of cecal content**

Cecal extracts were obtained from an aliquot from the cecum with 4 ml buffer (0.1% (w/v) HgCl₂ and 1% (v/v) H₂PO₄) containing 0.045 mg/ml 2,2-dimethylbutyric acid (as an internal standard) per gram fresh weight. The resulting slurry was centrifuged for 30 min at 5000 g at 4 °C and the supernatant containing SCFAs was analyzed using a gas chromatograph (HP 6890) equipped with flame ionization detector and a DB-FAP column (J&W Scientific, MSP Friedli & Co., Switzerland) of 30 m length, 530 μm diameter and 1 μm film thickness. The system was run with helium gas at an inlet constant pressure of 10 psi at 180 °C. A cleaning injection of 1.2% formic acid was used before each analysis. Samples were run at an initial temperature of 80 °C for 1.2 min followed by heating to 145 °C in 6.5 min, heating to 200 °C in 0.55 min and an additional 0.5 min at 200 °C. SCFAs were identified and quantified using the internal standard as well as external standards consisting of acetate, propionate, isobutyrate, n-butyrate, isovalerate and n-valerate.

**1H NMR spectroscopic analysis of biofluids and extracts**

Plasma samples (100 μl) were introduced into a 5 mm NMR tube with 450 μl of saline solution containing 10% D₂O as the locking substance. Urine samples were prepared by mixing 20 μl of samples with 30 μl of a phosphate buffer solution containing 90% D₂O and 0.25 mM 3-trimethylsilyl-1,2,3,3-tetramethylsilane (TSP), which was used as a reference.
a chemical shift reference, into 1.7 mm NMR tubes. Fecal pellets were homogenized in 650 μl of a phosphate buffer solution containing 90% D₂O and 0.25 mM TSP. The homogenates were sonicated at ambient temperature (298 K) for 30 min to destroy bacterial cells and then centrifuged at 6000 g for 20 min. The supernatants were removed and centrifuged again at 6000 g for 30 min. Aliquots of 0.55 μl were then pipetted into 5 mm NMR tubes. Intact liver samples were bathed in an ice-cold saline D₂O solution. A portion of the tissue (~ 15 mg) was packed into a zirconium oxide 4 mm outer-diameter rotor. All ¹H NMR spectra were recorded on a Bruker DRX 600 NMR spectrometer (Bruker Biospin, Rheinstetten, Germany) operating at 600.11 MHz for ¹H observation. ¹H NMR spectra of plasma, urine and fecal extracts were acquired with a Bruker 5 mm TXI triple-resonance probe at 298 K. ¹H NMR spectra of intact liver tissues were acquired using a standard Bruker high-resolution MAS probe under magic-angle-spinning conditions at a spin rate of 5000 Hz (Waters et al., 2000). Tissue samples were regulated at 283 K using cold N₂ gas to minimize any time-dependent biochemical degradation. ¹H NMR spectra of urine and fecal extracts were acquired using a standard one-dimensional pulse sequence (D1-90°-t1-90°-tm-90°-free induction decay (FID)). NMR spectra of plasma and tissues were acquired using the Carr-Purcell-Meiboom-Gill (CPMG, D1-90°-t180°-t2-FID) spin-echo pulse sequence with water suppression. Standard spectra were acquired with a relaxation delay D1 of 2 s during which the water resonance was selectively irradiated, and a fixed interval t1 of 3 μs. The water resonance was irradiated for a second time during the mixing time tm of 100 ms. CPMG spin-echo spectra were registered using a spin-echo loop time (2t1C) of 160 ms for plasma and 200 ms for tissue (Meiboom and Gill, 1958) and a relaxation delay of 2.5 s. A total of 128 and 256 transients were collected into 32K data points for standard and CPMG spectra respectively, with a spectral width of 20 ppm. The FIDs were multiplied by an exponential weighting function corresponding to a line broadening of 0.3 Hz. The acquired NMR spectra were manually phase- and baseline-corrected using the software package XwinNMR 3.5 (Bruker Biospin), and referenced to the chemical shift of the methyl resonance of alanine at δ 1.466 for plasma and tissue spectra and that of TSP at δ 0.00 for urine and fecal extract samples.

For assignment purposes, 2D COrelated Spectroscopy (COSY) (Nagayama, 1980) and 1D Total Correlation Spectroscopy (TOCSY) (Bax and Davis, 1985) NMR spectra were acquired on selected samples using a Bruker AV 400 spectrometer operating at 400.13 MHz for ¹H observation equipped with a Bruker 5 mm SEI (¹H-¹H) inverse probe with a z-axis field gradient coil at 298 K. Further assignment of the metabolite peaks was also accomplished with the use of TOCSY on 1D spectra.

Multivariate statistical analysis and visualization

Statistical analysis of the changes in animal weights, bacterial populations and in theecal composition of SCFAs obtained by GC was carried out using a two-tailed Mann–Whitney test. The ¹H NMR spectra were converted into 22K data points over the range of δ 0.2–10.0 using an in-house-developed MATLAB routine. The regions containing the water resonance (δ 4.5–5.19) and, for urine spectra, especially, the urea resonance (δ 4.5–6.2) were removed. The spectra were normalized to a constant total sum before chemometric analyses. The multivariate pattern recognition techniques used in this study were based on the O-PLS-DA approach with unit-variance scaling (Trygg and Wold, 2003). The O-PLS-DA loadings plots were processed according to the method described by Cloarec et al. (2005b). Here, the test for the significance of Pearson product-moment correlation coefficient was used to calculate the cutoff value of the correlation coefficients at the level of P < 0.05. To test the validity of the model against overfitting, the cross-validation parameter Q² was computed and the standard seven-fold cross-validation method was used (Cloarec et al., 2005b). Additional validation of the statistical modeling on urine, liver, feces and bacterial counts was performed using permutation testing based on cross-model validation methods recently published by Westerhuis et al. (2008). The means of the distributions of the Q² parameters obtained using random permutations are significantly different and lower than the experimental Q² parameters at 95% confidence interval using a one-tailed t-test. These data provide compelling evidence of the statistical validity of the models generated, and the results are provided as Supplementary information (Supplementary Figures 4 and 5).

Pixel map representation of the inter-compartment metabolic correlation

A statistical correlation analysis was applied to normalize the intensities of spectral peaks found to be significantly different with nutritional intervention to establish possible association between metabolites across different biological compartments. Pearson’s correlation coefficients were computed between influential metabolite relative intensities derived from liver and fecal metabolic profiles from the same mice within each group of mice colonized with one type of probiotic. Pixel maps were used to display the correlation matrices, and a cutoff value of 0.4 was applied to the absolute value of the coefficient |r| so that the map represents only those correlations between two metabolites that are above the cutoff. The value and the sign of the correlation were then color-coded (gradient of red colors for positive values, gradient of blue colors for negative values). The presence of colored pixels between specific metabolites reveals a correlation (above the cutoff) between these molecules that may reflect a functional association.

Bipartite graph representation of connectivities between SCFAs and microbial profiles

The bipartite graph (rigraphviz) package from R (Free Software Foundation General Public License, USA, Version 2.5.1) was used to display the correlation matrix derived from cecal SCFAs and microbial profiles (jejunal and fecal) to assess the prebiotic-induced changes in the microbial metabolism. Pearson’s correlation coefficients were computed between cecal SCFAs variables and microbiota variables from the same mice and a cutoff value of 0.5 was applied to the absolute value of the coefficient |r| so that the bipartite graph represents only those correlations between the two types of nodes (microbiota and SCFAs) that are above the cutoff (Martin et al., 2007a). The sign of the initial correlation was then color-coded (red positive, blue negative) and the correlation value displayed on the bipartite graph. In that context, presence of edges between two specific nodes (one of each type) reveals a correlation (above the cutoff) between these entities that may reflect a functional association.

Supplementary information

Supplementary information is available at the Molecular Systems Biology website (www.nature.com/msb).

Acknowledgements

We acknowledge the help and input of Ivan Mentoloi Roura, Olivier Cloarec and Marc-Emmanuel Dumas for statistical analysis; Isabelle Rochat, Catherine Murset and Gloria Reuteler for microbial analysis; and Rodrigo Bibiloni and Enea Rezzonico for helpful discussions on gut bacterial metabolism. We thank John Newell, Monique Julita, Massimo Marchesini, Catherine Schwartz and Christophe Maubert for provision of the animal facilities and expertise. This work received financial support from Nestlé (to F-PJM, YW) and from the International Study of Macro/micronutrient and Blood Pressure grant 1-R01-HL084228-01A1 (to IKSV).

References

Amaretti A, Bernardi T, Tamburini E, Zanoni S, Lomma M, Matteuzzi D, Rossi M (2007) Kinetics and metabolism of Bifidobacterium adolescentis MB 239 growing on glucose, galactose, lactose, and galactooligosaccharides. Appl Environ Microbiol 73: 3637–3644

© 2008 EMBO and Nature Publishing Group
Backhed F, Ding H, Wang T, Hooper LV, Koh GY, Nagy A, Semenovitch CF, Gordon JI (2004) The gut microbiota as an environmental factor that regulates fat storage. *Proc Natl Acad Sci USA* **101**: 15718–15723

Backhed F, Ley R, Sonnenburg J, Peterson D, Gordon J (2005) Host-bacterial mutualism in the human intestine. *Science* **307**: 1915–1920

Bax A, Davis D (1985) MLEV-17-based two-dimensional nuclear magnetic resonance spectroscopy. *J Magn Reson* **65**: 355–360

Bik EM, Eckburg PB, Gill SR, Nelson KE, Purdom EA, Francois F, Perez-Perez G, Blaser MJ, Relman DA (2006) Molecular analysis of the bacterial microbiota in the human stomach. *Proc Natl Acad Sci USA* **103**: 732–737

Blaut M, Cavel T (2007) Metabolic diversity of the intestinal microbiota: implications for health and disease. *J Nutr* **137**: 751S–755S

Bode L (2006) Recent advances on structure, metabolism, and function of human milk oligosaccharides. *J Nutr* **136**: 2127–2130

Cloarec O, Dumas ME, Craig A, Barton RH, Trygg J, Hudson J, Blanche C, Gauguidier D, Lindon JC, Holmes E, Nicholson J (2005a) Statistical total correlation spectroscopy: an exploratory approach for latent biomarker identification from metabolic 1H NMR data sets. *Anal Chem* **77**: 1282–1289

Cloarec O, Dumas ME, Trygg J, Craig A, Barton RH, Lindon JC, Nicholson JK, Holmes E (2005b) Evaluation of the orthogonal projection on latent structure model limitations caused by chemical shift variability and improved visualization of biomarker changes in 1H NMR spectroscopic metabonomic studies. *Anal Chem* **77**: 517–526

Collins MD, Gibson GR (1999) Probiotics, prebiotics, and symbiotics: approaches for modulating the microbial ecology of the gut. *Am J Clin Nutr* **69**: 1052S–1057S

Cuisinier C, Ward RJ, Francaux M, Sturbois X, de Witte P (2001) Changes in plasma and urinary taurine and amino acids in runners immediately and 24 h after a marathon. *Am J Physiol: Cell Physiol* **281**: C13–23

Delzenne NM, Kok N (1998) Effect of non-digestible fermentable carbohydrates on hepatic fatty acid metabolism. *Biochem Soc Trans* **26**: 228–230

Delzenne NM, Kok N (2001) Effects of fructans-type prebiotics on lipid metabolism. *Am J Clin Nutr* **73**: 456S–458S

Dehliøs L, Fall-Ngai M, Relman DA (2007) An ecological and evolutionary perspective on human–microbe mutualism and disease. *Nature* **449**: 811–818

Djouzi Z, Andrieux C (1997) Compared effects of three oligosaccharides on metabolism of intestinal microflora in rats inoculated with a human faecal flora. *Br J Nutr* **78**: 313–324

Djouzi Z, Andrieux C, Pelenc V, Somarrib S, Popot F, Paul F, Monsan P, Syltit O (1995) Degradation and fermentation of alpha-gluco-oligosaccharides by bacterial strains from human colon: an in vitro and in vivo studies in gnotobiotic rats. *J Appl Microbiol* **79**: 117–127

Dumas ME, Barton RH, Toy C, Cloarec O, Blanche C, Rothwell A, Fearnsd J, Tatoud R, Blanc V, Lindon JC, Mitchell SC, Holmes E, McCarthy MJ, Scott J, Gauguidier D, Nicholson JK (2006) Metabolic profiling reveals a contribution of gut microbiota to fatty liver phenotype in insulin-resistant mice. *Proc Natl Acad Sci USA* **103**: 12511–12516

Eckburg PB, Relman DA (2007) The role of microbes in Crohn’s disease. *Clin Infect Dis* **44**: 256–262

Ellis DI, Dunn WB, Griffin JL, Allwood JW, Goodacre R (2007) Metabolic fingerprinting as a diagnostic tool. *Pharmaconics* **8**: 1243–1266

Fioridaiolo M, Kok N, Desager JP, Goethals F, Deboysy D, Roberfroid M, Delzenne N (1995) Dietary oligofructose lowers triglycerides, phospholipids and cholesterol in serum and very low density lipoproteins of rats. *Lipids* **30**: 163–167

Gibson GR, Roberfroid MB (1995) Dietary modulation of the human colonic microbiota: introducing the concept of prebiotics. *J Nutr* **125**: 1401–1412

Gill SR, Pop M, Deboy RT, Eckburg PB, Turnbaugh PJ, Samuel BS, Gordon JI, Relman DA, Fraser-Liggett CM, Nelson KE (2006) Metagenomic analysis of the human distal gut microbiome. *Science* **312**: 1355–1359

Girard J, Ferre P, Foufelle F (1997) Mechanisms by which carbohydrates regulate expression of genes for glycolytic and lipogenic enzymes. *Annu Rev Nutr* **17**: 325–352

Goodacre R (2007) Metabolomics of a superorganism. *J Nutr* **137**: 2595–2668

Goodwin BL, Ruthven CR, Sandler M (1994) Gut flora and the origin of some urinary aromatic phenolic compounds. *Biochem Pharmacol* **47**: 2294–2297

Gopal PK, Prasad J, Gill HS (2003) Effects of the consumption of *Bifidobacterium lactis* HNO19 (DR10) (TM) and galacto-oligosaccharides on the microflora of the gastrointestinal tract in human subjects. *Nutr Res* **23**: 1313–1328

Gopal PK, Sullivan PA, Smart JB (2001) Utilisation of galacto-oligosaccharides as selective substrates for growth by lactic acid bacteria including *Bifidobacterium lactis* DR10 and *Lactobacillus rhamnosus* DR20. *Int Dairy J* **11**: 19–25

Griffin JL, Nicholls AW (2006) Metabolomics as a functional genomic tool for understanding lipid dysfunction in diabetes, obesity and related disorders. *Pharmacogenomics* **7**: 1095–1107

Guigoz Y, Rochat F, Perrusseaux-Carrier G, Rochat I, Schiffrin E (2002) Effects of oligosaccharide on the faecal flora and non-specific immune system in the elderly people. *Nutr Res* **22**: 13–25

Hallson PC, Choong SK, Kasidas GP, Samuell CT (1997) Effects of Tamm-Horsfall protein with normal and reduced sialic acid content upon the crystallization of calcium phosphate and calcium oxalate in human urine. *Br J Urol* **80**: 533–538

Hidaka H, Hirayama M (1991) Useful characteristics and commercial applications of fructo-oligosaccharides. *Biochem Soc Trans* **19**: 561–565

Holmes E, Nicholson J (2005) Variation in gut microbiota strongly influences individual rodent phenotypes. *Toxicol Sci* **87**: 1–2

Hopkins MJ, Cummings JH, McFarlane GT (1998) Interspecies differences in maximum specific growth rates and cell yields of bifidobacteria cultured on oligosaccharides and other simple carbohydrate sources. *J Appl Microbiol* **85**: 381–386

Houten SM, Watanabe M, Auwerx J (2006) Endocrine functions of bile acids. *EMBO J* **25**: 1419–1425

Ito M, Deguchi Y, Matsumoto K, Kimura M, Onodera N, Yajima T (1993) Influence of galactooligosaccharides on the human fecal microflora. *J Nutr Sci Vitamino (Tokyo)* **39**: 635–640

Katsurada A, Iritani N, Fukuda H, Matsumura Y, Nishimoto N, Nomuchi T, Tanaka T (1990) Effects of nutrients and hormones on transcriptional and post-transcriptional regulation of fatty acid synthase in rat liver. *Eur J Biochem* **190**: 427–433

Kikutk H, Andrieux C, Sylit O (1992) Effects of galacto-oligosaccharides on bacterial enzymatic activities and metabolite production in rats associated with a human flora. *Proc Nutr Soc* **51**: 7A

Kliever SA, Sundsset SS, Jones SA, Brown PJ, Wisely GB, Kobl S, Devchand P, Wahli W, Willson TM, Lehmann JM (1997) Fatty acids and eicosanoids regulate gene expression through direct interactions with peroxisome proliferator-activated receptors alpha and gamma. *Proc Natl Acad Sci USA* **94**: 4318–4323

Kok N, Roberfroid M, Robert A, Delzenne N (1996) Involvement of lipogenesis in the lower VLDL secretion induced by oligofructose in rats. *Br J Nutr* **76**: 881–890

Kok NN, Taper HS, Delzenne NM (1998) Oligofructose modulates lipid metabolism alterations induced by a fat-rich diet in rats. *J Appl Toxicol* **18**: 47–53
Kunz C, Rudloff S, Baiar W, Klein N, Strobel S (2000) Oligosaccharides in human milk: structural, functional and metabolic aspects. *Ann. Rev. Nutr.* 20: 699–722

Ley R, Turnbaugh P, Klein S, Gordon J (2006) Human gut microbes associated with obesity. *Nature* 444: 1023–1024

Lim CC, Ferguson LR, Tannock GW (2005) Dietary fibres as 'prebiotics': implications for colorectal cancer. *Mol Nutr Food Res.* 49: 609–619

Macfarlane GT, Gibson GR, Beatty E, Cummings JH (1992) Estimation of short-chain fatty-acid production from protein by human intestinal bacteria based on branched-chain fatty-acid measurements. *FEBS Microbiol Ecol.* 101: 81–88

Macfarlane GT, Steed H, Macfarlane S (2008) Bacterial metabolism and health-related effects of galacto-oligosaccharides and other prebiotics. *J Appl Microbiol.* 104: 305–344

Macfarlane S, Macfarlane GT, Cummings JH (2006) Review article: prebiotics in the gastrointestinal tract. *Aliment Pharmacol Ther.* 24: 701–714

Mackie RI, Sghir A, Gaskins HR (1999) Developmental microbial ecology of the neonatal gastrointestinal tract. *Am J Clin Nutr.* 69: 1035S–1045S

Martin FP, Dumas ME, Wang Y, Legido-Quigley C, Yap IK, Tang H, Zirah S, Murphy GM, Cloarec O, Lindon JC, Sprenger N, Fay LB, Kochhar S, van Bladeren P, Holmes E, Nicholson JK (2007a) A top-down systems biology view of microbiome–mammalian metabolic interactions in a mouse model. *Mol Syst Biol.* 3: 112

Martín FP, Verdu EF, Wang Y, Dumas ME, Yap IK, Cloarec O, Bergonzelli GE, Cortes-Venzon I, Kochhar S, Lindon JC, Collins SM, Nicholson JK (2006) Transgenomic metabolic interactions in a mouse disease model: interactions of *Trichinella spiralis* infection with dietary *Lactobacillus paracasei* supplementation. *J Proteome Res.* 5: 2185–2193

Martin FP, Wang Y, Sprenger N, Holmes E, Lindon JC, Kochhar S, Nicholson JK (2007b) Effects of probiotic *Lactobacillus paracasei* treatment on the host gut tissue metabolic profiles probed via magic-angle-spinning NMR spectroscopy. *J Proteome Res.* 6: 1471–1481

Martin FP, Wang Y, Sprenger N, Yap IK, Lundstedt T, Lek P, Rezzi S, Ramadan Z, van Bladeren P, Fay LB, Kochhar S, Lindon JC, Holmes E, Nicholson JK (2008) Probiotic modulation of symbiotic gut microbial-host metabolic interactions in a humanized microbiome mouse model. *Mol Syst Biol.* 4: 157

Meiboom S, Gill D (1958) Modified spin-echo method for measuring nuclear relaxation times. *Rev Sci Instrum.* 29: 688–691

Mecas CC (2000) Contribution of microbial amino acids to amino acid homeostasis of the host. *J Nutr.* 130: 1857S–1864S

Mitsuoka T, Hidaka H, Eida T (1987) Effect of fructo-oligosaccharides on gut flora metabolism in rats associated with a human fecal microflora. *J Appl Bacteriol.* 74: 667–674

Roberfroid MB, Delzenne NM (1998) Dietary fructans. *Annu Rev Nutr.* 18: 117–143

Rowland I (1993) The effects of transgalactosylated oligosaccharides on gut flora metabolism in rats associated with a human fecal microflora. *J Appl Bacteriol.* 74: 667–674

Rycoct CE, Jones MR, Gibson GR, Rastall RA (2001) A comparative in vitro evaluation of the fermentation properties of prebiotic oligosaccharides. *J Appl Microbiol.* 91: 878–887

Sartor RB (2004) Therapeutic manipulation of the enteric microbiota in inflammatory bowel diseases: antibiotics, probiotics, and prebiotics. *Gastroenterology* 126: 1620–1633

Schell MA, Karmiriantzou M, Snel B, Vilanova D, Berger B, Pessi G, Zawahlein MC, Desiere F, Bork P, Delley M, Pridmore RD, Arigoni F (2002) The genome sequence of *Bifidobacterium longum* reflects its adaptation to the human gastrointestinal tract. *Proc Natl Acad Sci USA.* 99: 14422–14427

Schrezenmeir J, de Vrese M (2001) Prebiotics, probiotics, and symbiotics—approaching a definition. *Am J Clin Nutr.* 73: 361S–364S

Smith EA, Macfarlane GT (1996) Enumeration of human colonic bacteria producing phenolic and indolic compounds: effects of pH, carbohydrate availability and retention time on dissimilatory aromatic amino acid metabolism. *J Appl Bacteriol.* 81: 288–302

Smith JL, Wishnok JS, Deen WM (1994) Metabolism and excretion of methylamines in rats. *Toxicol Appl Pharmacol.* 125: 296–308

Sonnenburg JL, Chen CT, Gordon JI (2006) Genomic and metabolic studies of the impact of probiotics on a model gut symbiont and host. *PLoS Biol.* 4: e141

Stanley JC, Newsholme EA (1985) The effect of dietary guar gum on the activities of some key enzymes of carbohydrate and lipid metabolism in mouse liver. *Br J Nutr.* 53: 215–222

Stella C, Beckwith-Hall B, Cloarec O, Holmes E, Lindon JC, Powell J, van der Ouderaa F, Bingham S, Cross AJ, Nicholson JK (2006) Susceptibility of human metabolic phenotypes to dietary modulation. *J Proteome Res.* 5: 2780–2788

Tannock GW (2004) A special fondness for lactobacilli. *Rev Nutr.* 26: 259–281

Tannock GW (2005) Commentary: remembrance of microbes past. *Int J Epidemiol.* 34: 13–15

Trypt J, Wold S (2003) 02-PLS, a two-block (X–Y) latent variable regression (LVR) method with an integrated OSC filter. *J Chemom.* 17: 53–64

Turnbaugh P, Ley R, Mahowald M, Magrini V, Mardis E, Gordon J (2006) An obesity-associated gut microbiome with increased capacity for energy harvest. *Nature.* 444: 1027–1031

Turnbaugh PJ, Ley RE, Hamady M, Fraser-Liggett CM, Knight R, Gordon JI (2007) The human microbiome project. *Nature.* 449: 804–810

Tzortzis G, Goulas AK, Gee JM, Gibson GR (2005) A novel galactooligosaccharide mixture increases the bifidobacterial population numbers in a continuous in vitro fermentation system and in the proximal colonic contents of pigs in vivo. *J Nutr.* 135: 1726–1731

Wang Y, Lawler D, Larson B, Ramadan Z, Kochhar S, Holmes E, Nicholson JK (2007) Metabonomic Investigations of aging and caloric restriction in a life-long dog study. *J Proteome Res.* 6: 1846–1854

Wang Y, Tang H, Nicholson JK, Hylands PJ, Sampson J, Holmes E (2005) A metabonomic strategy for the detection of the metabolic effects of chlamomile (*Matricaria recutita* L.) ingestion. *J Agric Food Chem.* 53: 191–196

Watanabe M, Houten SM, Mataki C, Christofleota MA, Kim BW, Sato H, Messaddeq N, Harney JW, Ezaki O, Kodama T, Schoonjans K, Bianco AC, Auwerx J (2006) Bile acids induce energy expenditure by promoting intracellular thyroid hormone activation. *Nature.* 439: 484–489

Watkins NJ, Garrod S, Farrant RD, Haselden JN, Connor SC, Connelly J, Lindon JC, Holmes E, Nicholson JK (2000) High-resolution magic angle spinning 1H NMR spectroscopy of intact liver and kidney: Bile acid metabolism and personalized health care. *Nat Rev Drug Discov.* 1: 69–79

Prebiotics modulation of mammalian metabolism

F-PJ Martin et al
optimisation of sample preparation procedures and biochemical stability of tissue during spectral acquisition. *Anal Biochem* **282**: 16–23

Westerhuis JA, Hoefsloot HC, Smit S, Vis DJ, Smilde AK, van Velzen EJ, van Duijnhoven JP, van Dorsten FA (2008) Assessment of PLSDA cross validation. *Metabolomics* **4**: 81–89

Xu J, Bjursell MK, Himrod J, Deng S, Carmichael LK, Chaing HC, Hooper LV, Gordon JI (2003) A genomic view of the human–*Bacteroides thetaiotaomicron* symbiosis. *Science* **299**: 2074–2076

Zeisel SH, Wishnok JS, Blusztajn JK (1983) Formation of methylamines from ingested choline and lecithin. *J Pharmacol Exp Ther* **225**: 320–324

*Prebiotics modulation of mammalian metabolism* F-PJ Martin et al

Molecular Systems Biology is an open-access journal published by European Molecular Biology Organization and Nature Publishing Group. This article is licensed under a Creative Commons Attribution-Noncommercial-No Derivative Works 3.0 Licence.