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

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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.
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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*, 2007a).

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

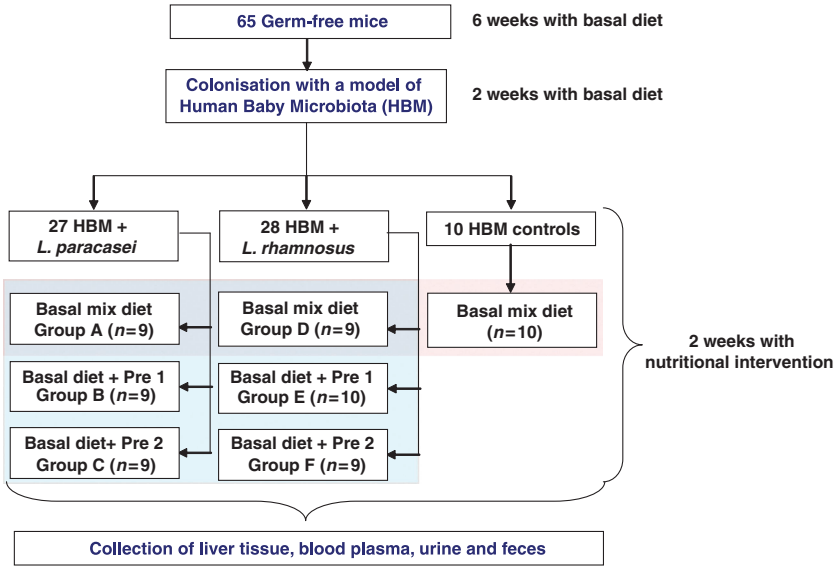

Figure 1 Schematic diagram of the experimental study design.

Table I 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	—	—	—	—	7.8 ± 0.2	7.6 ± 0.4	7.9 ± 0.3
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	7.78 ± 2.13	8.5 ± 1.5	8.7 ± 1.5	8.7 ± 0.3	9.8 ± 0.3**	10.0 ± 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.

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 microbiota as described previously (Martin *et al*, 2008). For clarity, two-dimensional representations of the data scores plots are given in Figure 2B and C along with the corresponding loadings plots (Figure 2D and E). Interestingly, the combination of *L. rhamnosus* and Pre2 formed a subcluster along the first component Tcv1 closer to the control HBM colonized group than the other nutritional intervention groups, and this was predominantly influenced by higher fecal *B. longum* and lower *C. perfringens* populations when compared with other groups. The effects of the two prebiotics (green triangles for Pre1 and blue diamonds for Pre2)

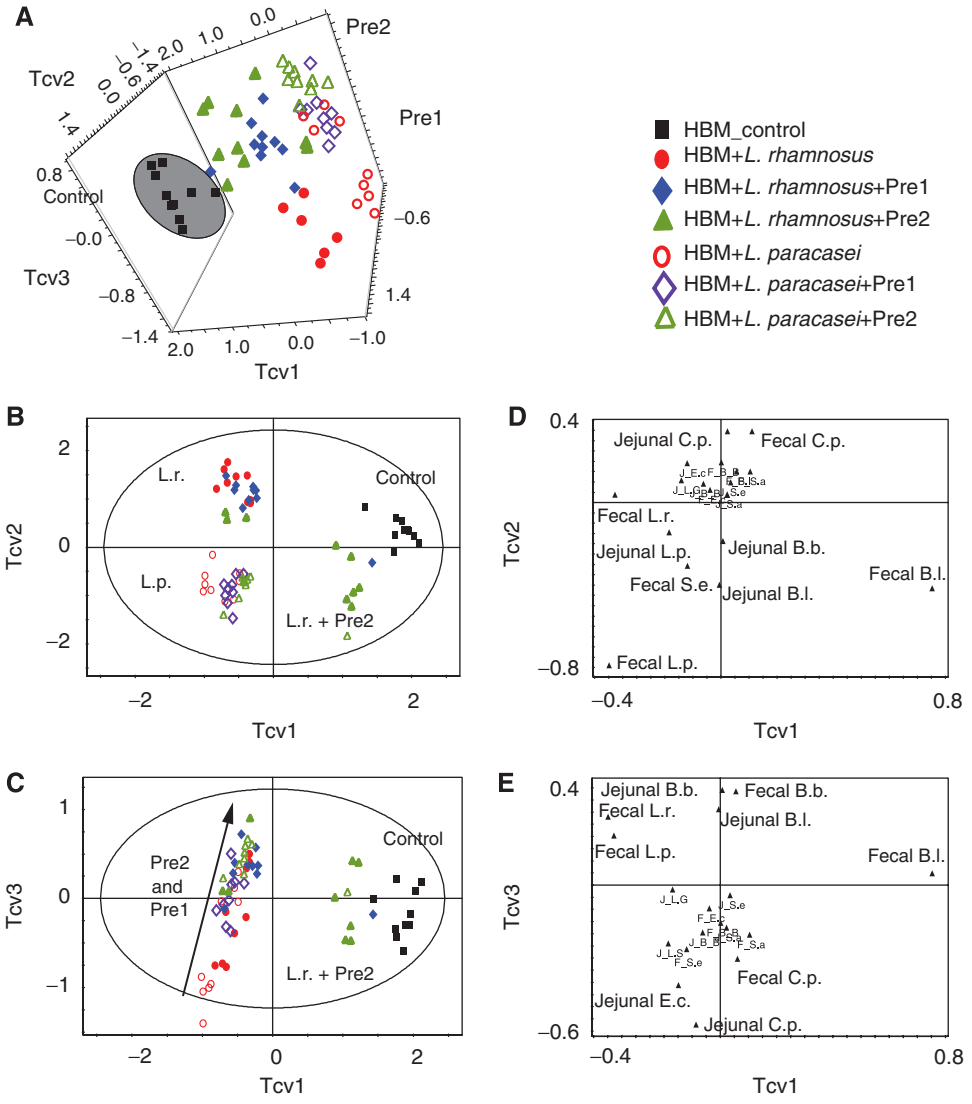

Figure 2 PLS-DA scores plots (**A**–**C**) and loading plots for the three predictive components (**D**, **E**) derived from PLS-DA model of $\log_{10}$ CFU (colony forming unit) for the different bacterial species measured for fecal and jejunal samples from HBM control (black square), HBM + *L. rhamnosus* (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_X^2=76.5%, Q_Y^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*.

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

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 ± 42.2 (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 μmol 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.

study, only minor changes in lactate were observed in similar experiments with a slight reduction in cecal lactate when feeding *L. rhamnosus* + Pre2 (unpublished data).

^{1}H NMR metabolic profiles of plasma, liver, fecal extracts and urine

A comprehensive ^{1}H nuclear magnetic resonance (NMR) spectral comparison was carried out using orthogonal projection to latent structure discriminant analysis (O-PLS-DA) to characterize metabolic changes associated with prebiotic supplementation and the metabolic changes were verified by two-dimensional NMR experiments as shown in Figure 3. Several amino acids, glucosides and SCFAs were readily assigned, as exemplified in Table III. Some ^{1}H-^{1}H cross-peaks at δ5.42:3.98, 5.52:3.79, 5.40:3.61 and 5.40:3.80 show values typical of short-chain oligosaccharides, which are as yet unidentified (Figure 3).

Goodness of fit (R_X^2) and predictability (Q_Y^2) values for O-PLS-DA models calculated separately for NMR spectroscopic data for plasma, urine, fecal extracts and intact liver tissues are presented in Table III. All the models were calculated using one predictive component and two orthogonal components using the NMR data as the *X* matrix and the type of prebiotics treatment (none, Pre1 or Pre2) as a dummy *Y* variable. No impact of prebiotic supplementation on the metabolic profiles of plasma was observed as indicated by the negative values of Q_Y^2 (Table III). However, prebiotic supplementation showed significant effects on murine metabolic profiles of urine, fecal extracts and liver, as reflected by the high values of Q_Y^2 for each model (Table III). The O-PLS-DA coefficients plots for models based on NMR spectra of fecal extracts, liver tissue and urine are presented in Figures 4 and Supplementary Figures 1 and 2 together with metabolites with the highest coefficient values responsible for the discriminatory variation listed as mean ± s.d. in Table III.

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 oligosaccharide resonances (O1, O3), which were associated with decreased levels of resonances derived from other oligosaccharides (O2) for Pre2 (Table III and Supplementary Figure 1). In HBM mice supplemented with *L. rhamnosus*, Pre1 and Pre2 induced some degree of reduction in the levels of unassigned oligosaccharides (O2). Pre2 treatment was also correlated with increases in the other unassigned oligosaccharides (O1 and O3).

Further O-PLS-DA models of a pairwise comparison between Pre1 and Pre2 showed clear differences in the content of oligosaccharides O1 and O3 between the groups of HBM mice supplemented with either *L. paracasei* or *L. rhamnosus* (Supplementary Figure 3A and B). The changes in the fecal content of oligosaccharides O1 and O3 may thus result from differences in the content of products obtained from the digestion of prebiotics by the gut microbiota.

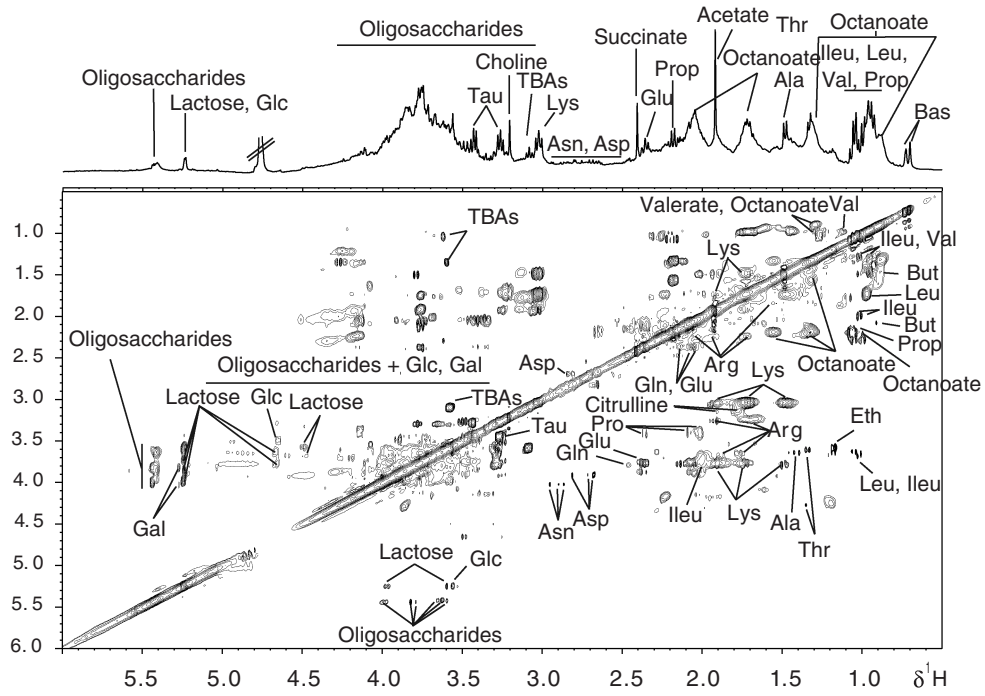

Figure 3 ^{1}H-^{1}H TOCSY NMR spectrum of a fecal extract acquired at 400 MHz. Key: Ala, alanine; Arg, arginine; Asn, asparagine; Asp, aspartate; BAs, bile acids; But, butyrate; Eth, ethanol; Gal, galactose; Glc, glucose; Gln, glutamine; Glu, glutamate; Ileu, isoleucine; Leu, leucine; Lys, lysine; Phe, phenylalanine; Pro, proline; Prop, propionate; Tau, taurine; TBAs, tauro-conjugated bile acids; Trp, tryptophan; Val, valine.

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 (^{1}H NMR chemical shifts: 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 phenylacetylglycine, *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 (^{1}H NMR chemical shift: 4.21(s)).

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

Table III Summary of influential metabolites for discriminating ^{1}H NMR spectra of liver, fecal extracts and urine

Metabolite	Chemical shift and multiplicity	HBM + *L.p.*	HBM + *L.p.* + Pre1	HBM + *L.p.* + Pre2	HBM + *L.r.*	HBM + *L.r.* + Pre1	HBM + *L.r.* + Pre2
Plasma			$Q_Y^2 < 0$	$Q_Y^2 < 0$		$Q_Y^2 < 0$	$Q_Y^2 < 0$
Liver			$Q_Y^2 = 52\%$, $R_X^2 = 49\%$	$Q_Y^2 = 57\%$, $R_X^2 = 57\%$		$Q_Y^2 = 38\%$, $R_X^2 = 39\%$	$Q_Y^2 = 49\%$, $R_X^2 = 38\%$
Glu	2.34(m)	0.4 ± 0.1	0.6 ± 0.2*	0.5 ± 0.1	0.5 ± 0.2	0.9 ± 0.2**	0.6 ± 0.1
Gln	2.44(m)	1.3 ± 0.4	1.8 ± 0.4*	2.0 ± 0.6*	1.6 ± 0.6	2.0 ± 0.6*	1.8 ± 0.4
TGL	1.27(s)	60.9 ± 13.5	42.7 ± 13.9*	43.4 ± 9.5**	44.8 ± 16.2	31.9 ± 11.9	42.6 ± 15.8
PUFAs	5.26(m)	0.2 ± 0.1	0.4 ± 0.1*	0.4 ± 0.1***	0.4 ± 0.1	0.5 ± 0.1*	0.4 ± 0.1
Ileu	0.94(t)	1.9 ± 0.3	2.4 ± 0.5*	2.4 ± 0.3*	2.3 ± 0.5	2.9 ± 0.6[a]	2.4 ± 0.3
Glycogen	5.45 (m)	1.1 ± 0.4	1.8 ± 1.0[a]	1.5 ± 0.4[a]	2.5 ± 1.2	2.1 ± 0.1	1.3 ± 0.5[a]
TMA	2.91 (s)	0.1 ± 0.04	0.2 ± 0.1**	0.2 ± 0.04***	0.2 ± 0.1	0.2 ± 0.04	0.2 ± 0.1
TMAO	3.27(s)	13.1 ± 3.7	14.3 ± 4.8	20.6 ± 4.8**	20.7 ± 5.1	18.2 ± 6.9	26.3 ± 9.8[a]
Gly	3.56(s)	3.4 ± 0.5	4.1 ± 1.1	4.7 ± 1.0*	5.2 ± 1.7	5.0 ± 1.7	5.0 ± 1.5
Ala	1.46(d)	6.4 ± 1.9	5.2 ± 1.9	6.7 ± 1.8	7.2 ± 2.3	8.0 ± 1.0	9.6 ± 3.5
PC	3.20(s)	17.6 ± 3.5	16.1 ± 2.1	20.8 ± 3.9	19.2 ± 4.3	22.5 ± 4.9	24.5 ± 5.4[a]
Feces			$Q_Y^2 = 87\%$, $R_X^2 = 41\%$	$Q_Y^2 = 87\%$, $R_X^2 = 40\%$		$Q_Y^2 = 85\%$, $R_X^2 = 49\%$	$Q_Y^2 = 91\%$, $R_X^2 = 43\%$
Oligosaccharides O3	3.67(m)	5.6 ± 0.2	6.1 ± 0.2***	6.8 ± 0.2***	5.9 ± 0.1	6.1 ± 0.1	7.1 ± 0.3***
Oligosaccharides O2	5.43(m)	1.2 ± 0.1	1.2 ± 0.1	0.9 ± 0.1***	1.6 ± 0.1	1.2 ± 0.2***	0.9 ± 0.1***
Oligosaccharides O1	3.94(m)	4.7 ± 0.2	5.3 ± 0.4**	5.7 ± 0.2***	4.8 ± 0.4	4.9 ± 0.4	6.1 ± 0.6***
Arg	1.66(m)	2.0 ± 0.1	2.7 ± 0.1***	2.1 ± 0.2	2.1 ± 0.1	2.8 ± 0.2***	1.9 ± 0.2
Citrulline	3.15(t)	1.4 ± 0.1	2.1 ± 0.1***	1.4 ± 0.1	1.4 ± 0.1	1.9 ± 0.2***	1.4 ± 0.1
Octanoate	1.27(m)	4.2 ± 0.7	2.4 ± 0.2***	3.8 ± 0.7	2.0 ± 0.2	2.5 ± 0.4***	3.8 ± 0.6***
Lys	3.02(m)	5.1 ± 0.3	5.4 ± 0.9	3.5 ± 0.4***	4.9 ± 0.1	4.4 ± 0.9	3.9 ± 0.6*
Butyrate	2.16(t)	4.4 ± 0.3	4.3 ± 0.3	3.8 ± 0.2***	4.6 ± 0.4	4.2 ± 0.6	3.7 ± 0.4**
Isovalerate	2.03(t)	4.3 ± 0.4	4.0 ± 0.3	3.5 ± 0.3***	4.1 ± 0.4	3.8 ± 0.2	3.5 ± 0.2**
Propionate	1.06(t)	4.1 ± 0.6	3.5 ± 0.7	2.8 ± 0.8**	2.9 ± 0.7	2.1 ± 0.5	2.2 ± 0.6
Glu	2.34(m)	3.0 ± 0.3	3.3 ± 0.3	3.1 ± 0.1	3.3 ± 0.2	2.8 ± 0.2***	2.9 ± 0.4**
Lactose	5.23(d)	1.9 ± 0.2	1.5 ± 0.2**	1.6 ± 0.3*	2.0 ± 0.1	1.3 ± 0.1***	1.7 ± 0.3*
Glucose	5.23(d)	1.3 ± 0.1	1.3 ± 0.1	1.1 ± 0.1*	1.6 ± 0.1	1.3 ± 0.1***	1.2 ± 0.1***
Urine			$Q_Y^2 = 66\%$, $R_X^2 = 50\%$	$Q_Y^2 = 72\%$, $R_X^2 = 47\%$		$Q_Y^2 = 63\%$, $R_X^2 = 45\%$	$Q_Y^2 = 81\%$, $R_X^2 = 50\%$
Creatinine	4.05(s)	15.0 ± 3.9	14.3 ± 2.9	10.8 ± 5.5	15.9 ± 2.4	11.9 ± 7.0	8.4 ± 1.9***
PAG	7.37(m)	1.5 ± 0.2	1.1 ± 0.3*	1.2 ± 0.5	1.2 ± 0.4	1.1 ± 0.3	1.1 ± 0.2
Tryptamine	7.70(d)	0.4 ± 0.1	0.2 ± 0.1*	0.2 ± 0.1	0.3 ± 0.2	0.2 ± 0.1	0.2 ± 0.1
ULp	1.27(m)	2.9 ± 0.5	1.9 ± 0.2**	2.2 ± 0.3*	1.8 ± 0.2	1.8 ± 0.3	2.8 ± 0.7***
Nac	2.06(s)	0.3 ± 0.1	0.2 ± 0.1*	0.2 ± 0.1	0.4 ± 0.2	0.2 ± 0.1	0.2 ± 0.1
1-NMN	4.48(s)	0.7 ± 0.1	1.4 ± 0.3***	1.6 ± 0.3**	1.1 ± 0.5	1.3 ± 0.7	1.1 ± 0.1
Creatine	3.92(s)	4.5 ± 1.5	4.8 ± 1.3	12.2 ± 6.0**	3.5 ± 0.3	9.9 ± 8.0**	8.2 ± 3.4***
Glycerate	4.04 (m)	5.1 ± 0.3	5.3 ± 0.3	7.2 ± 1.3*	5.2 ± 0.5	5.0 ± 0.3	5.4 ± 0.4
Citrate	2.55(d)	5.1 ± 2.1	10.4 ± 5.1*	6.6 ± 4.4	11.9 ± 8.5	7.4 ± 4.0	7.7 ± 4.2
TMA	2.91(s)	1.1 ± 0.1	1.4 ± 0.5	1.9 ± 0.6**	1.0 ± 0.2	1.0 ± 0.5	1.6 ± 0.3***
α-Keto-isovalerate	1.13(d)	4.4 ± 1.8	4.0 ± 2.1	2.1 ± 0.8[a]	3.7 ± 1.7	2.4 ± 1.3	1.8 ± 0.4*
α-Keto-glutarate	3.19(t)	4.1 ± 2.2	3.5 ± 2.5	1.8 ± 0.1	2.7 ± 1.9	3.1 ± 2.7	2.3 ± 0.1
Arg	1.66(m)	3.7 ± 0.5	3.4 ± 0.2	2.5 ± 0.3**	3.4 ± 0.2	2.7 ± 0.2***	2.3 ± 0.1***
Citrulline	1.88(m)	3.1 ± 0.5	2.9 ± 0.2	2.3 ± 0.4*	3.1 ± 0.6	2.4 ± 0.2*	2.1 ± 0.2***
Taurine	3.44(t)	10.7 ± 8.6	10.5 ± 5.0	9.8 ± 5.3	9.0 ± 7.2	31.8 ± 12.6**	17.8 ± 10.4*
U1	4.30(t)	1.6 ± 0.3	2.0 ± 0.3*	1.9 ± 0.2[a]	1.7 ± 0.2	2.7 ± 0.9**	2.2 ± 0.5*
U2	4.21(s)	1.5 ± 0.2	1.5 ± 0.2	1.9 ± 0.4[a]	1.2 ± 0.1	1.6 ± 0.6	2.2 ± 1.0***

Data are presented as area-normalized intensities (10^1 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.
Key: s, singlet; d, doublet; t, triplet; q, quartet; m, multiplet; dd, doublet of doublets. Ala, alanine; Arg, arginine; Gln, glutamine; Glu, glutamate; Gly, glycine; Ileu, isoleucine; Leu, leucine; Lys, lysine; PUFAs, polyunsaturated fatty acids; PC, phosphocholine; TGL, triglycerides; TMA, trimethylamine; TMAO, trimethylamine-*N*-oxide. For abbreviations of other metabolites, refer to keys in Figures 3 and 4.

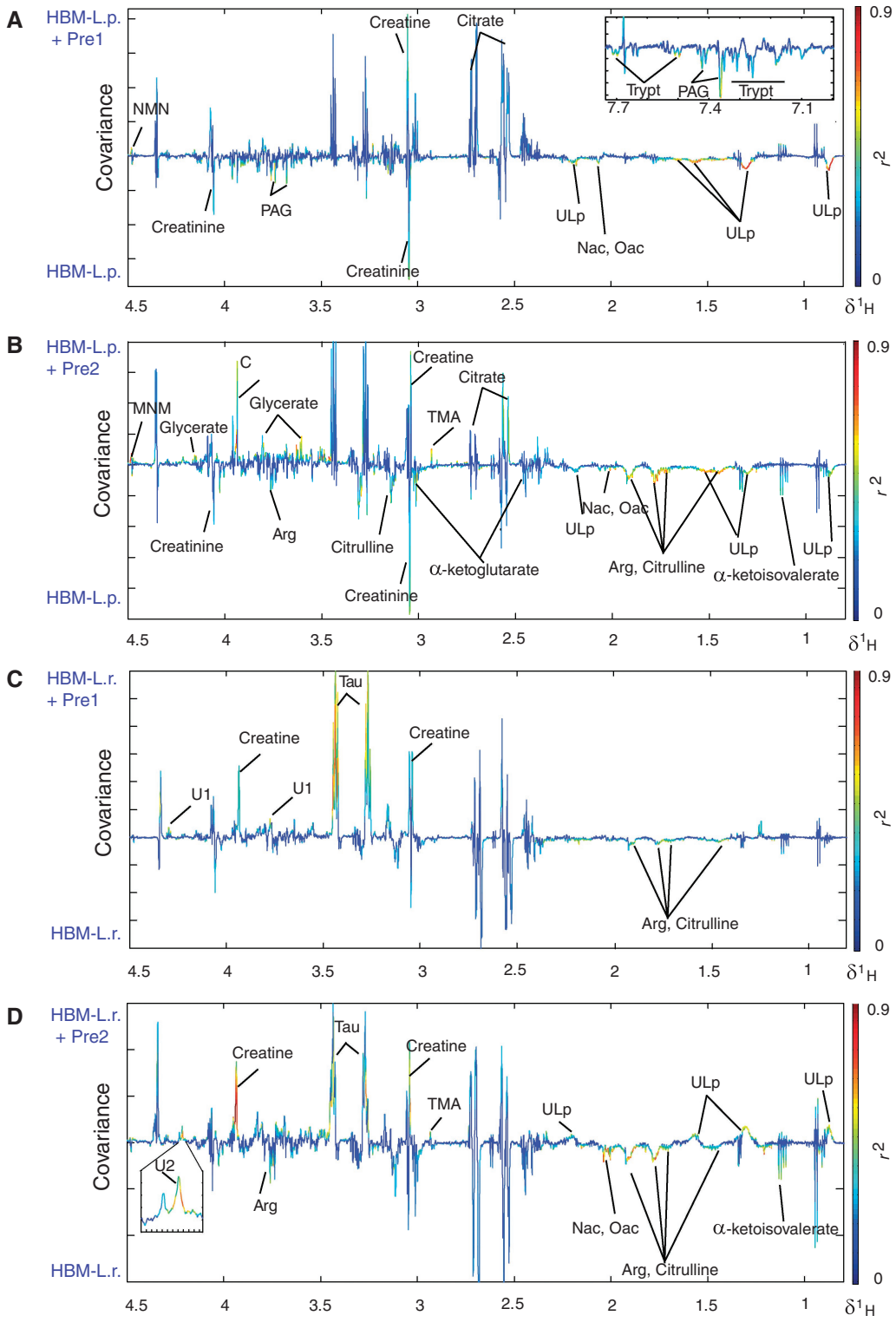

Figure 4 O-PLS-DA coefficients for a model derived from ^{1}H NMR spectra of urine based on the discrimination between HBM mice fed with probiotics only (negative) and HBM mice fed with probiotics and prebiotics (positive): *L. paracasei* supplementation with and without prebiotics Pre1 and Pre2 is shown in (**A**) and (**B**), whereas *L. rhamnosus* supplementation with and without prebiotics Pre1 and Pre2 is shown in (**C**) and (**D**). Key: Arg, arginine; GPC, glycerophosphorylcholine; Nac, *N*-acetylated glycoproteins; MNM, 1-methylnicotinamide; PAG, phenylacetylglycine; Tau, taurine; TMA, trimethylamine; Trypt, tryptamine; ULp, unidentified lipids.

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

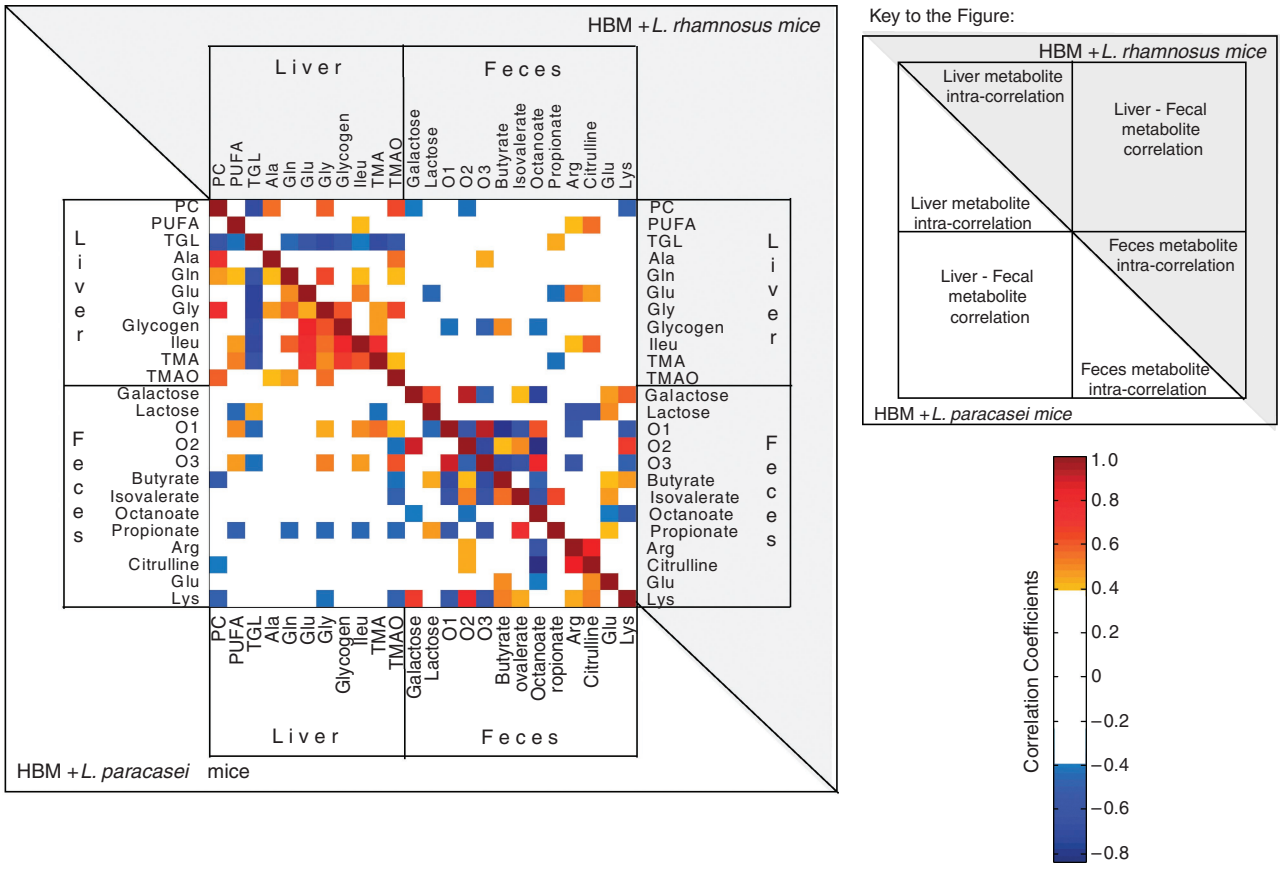

Figure 5 Integration of inter-compartment metabolic correlations. The pixel maps were derived from correlations between liver and fecal metabolites found to be significantly different with nutritional intervention in each group of mice colonized with one type of probiotic. The intra- and inter-compartmental 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*| for displaying the correlations between metabolites. 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.

differences previously described. In HBM mice receiving *L. paracasei* supplementation, hepatic PUFAs and isoleucine showed positive associations with the oligosaccharide resonances O1 and O3, whereas hepatic triglycerides were negatively correlated to O1 and O3. These data suggested a direct relationship between carbohydrate digestion and liver lipid metabolism. These metabolic links were not observed in the matrix of correlations obtained from HBM + *L. rhamnosus*. Interestingly, negative correlations between O1 and O3 with fecal SCFAs illustrate bacterial fermentation of dietary carbohydrates in both groups. The pixel map also highlighted the positive correlations between glucogenic amino acids, glycogen and PUFAs in the liver, suggesting functional relationships between glucogenesis and gluconeogenesis.

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

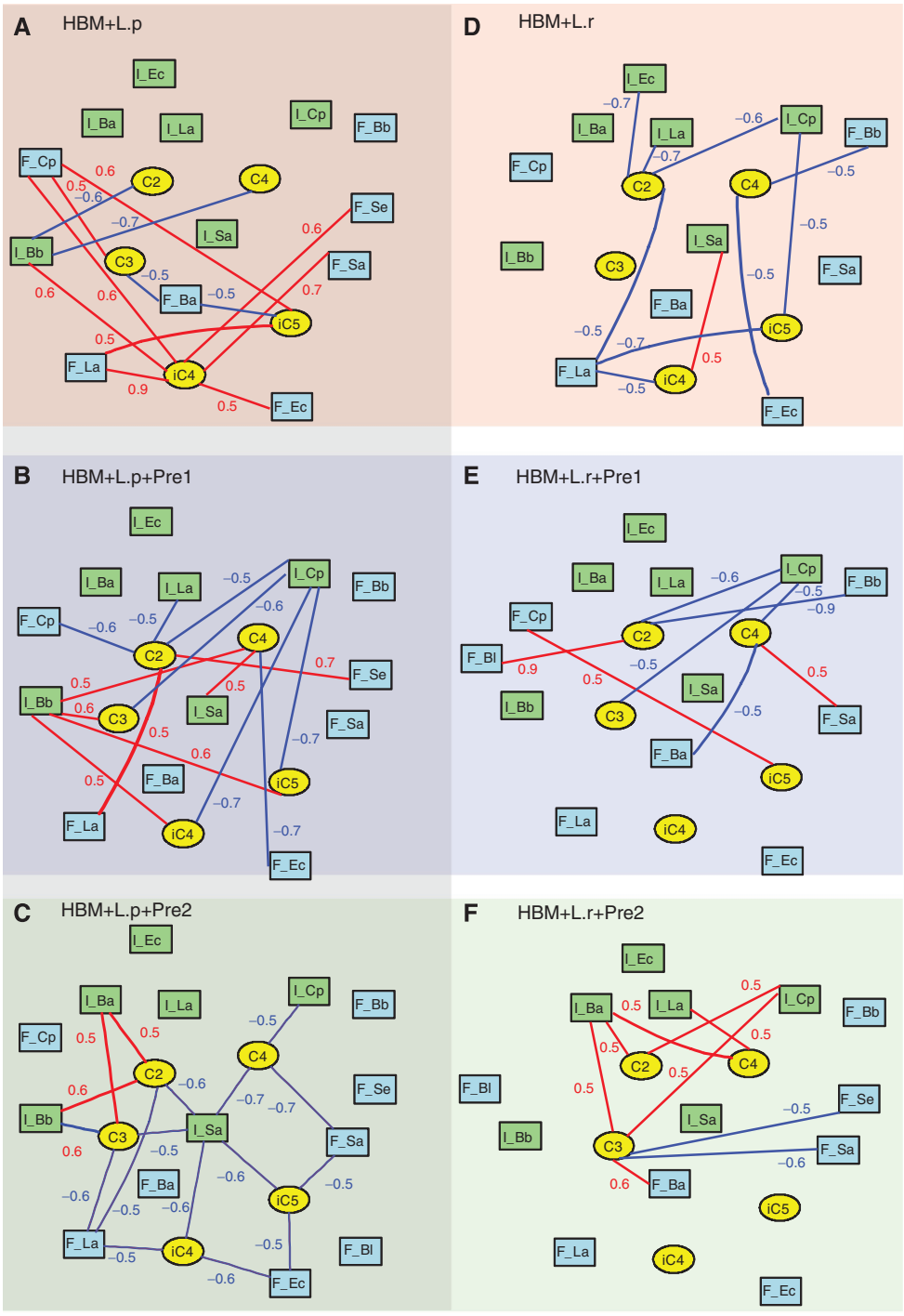

Figure 6 Integration of SCFAs and microbiota correlations. The bipartite graphs were derived from correlations between microbiota (fecal and jejunal) and SCFAs in each of the six groups of mice. HBM mice were supplemented with *L. paracasei* without prebiotics (**A**), with Pre1 (**B**) and with Pre2 (**C**), and HBM mice were supplemented with *L. rhamnosus* (**D**), with Pre1 (**E**) and with Pre2 (**F**). The cutoff value of 0.5 was applied to the absolute value of the coefficient |*r*| for displaying the correlations between microbiota and SCFAs. SCFAs, and intestinal and fecal bacteria correspond to yellow ellipse nodes, and blue and green rectangle nodes, respectively. Edges are coded according to correlation value: positive and negative correlations are displayed in blue and red, respectively. Key: Ba., *Bacteroides distasonis*; Bb., *Bifidobacterium breve*; Bl, *Bifidobacterium longum*; C2, acetate; C3, propionate; C4, butyrate; iC4, isobutyrate; C5, valerate; iC5, isovalerate; Cp, *Clostridium perfringens*; Ec, *Escherichia coli*; La, *Lactobacilli probiotics*; Sa, *Staphylococcus aureus*; Se, *Staphylococcus epidermidis*. Bacterial names starting with 'F_' and 'I_' correspond to fecal and intestinal bacteria, respectively.

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 *C. perfringens* and *B. breve*, respectively (Figure 6B). Pre2 supplementation was associated with positive correlations between SCFAs (acetate and propionate) and both *Bacteroides distasonis* and *B. breve* (Figure 6C). In contrast, negative correlations were observed for *S. aureus* with most of the SCFAs (Figure 6C).

When HBM mice received *L. rhamnosus* with or without prebiotics, the microbiome/SCFAs network showed a simpler structure. Such observations suggest different bacterial interactions in both HBM + probiotic models. Available microbial data support this idea as seen, for instance, with the specific inhibition of *B. breve* growth with *L. paracasei* but not with *L. rhamnosus*. In contrast with animals supplemented with *L. paracasei* alone, variation in the intestinal bacterial populations from mice that received *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 *B. breve*, *B. longum* and *C. perfringens*. Interestingly, Pre2 appeared to initiate functional relationships between the main SCFAs (acetate, propionate and butyrate) and *Bacteroides distasonis*, *C. perfringens* and lactobacilli.

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 glucogenic amino acids. These data provide further evidence for the critical involvement of prebiotics in host metabolism through modulation of the gut microbiome.

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 (*Clostridium* spp, *Staphylococcus* spp and the members of the Enterobacteria and 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 *B. longum* and *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 *L. rhamnosus* and *L. paracasei* were not able to grow on culture media containing Pre1, whereas *B. breve* and *B. longum* did not show any difference in growth when cultured on Pre1, glucose or lactose media (unpublished data). In addition, the ability of *B. longum* (Hopkins *et al*, 1998; Macfarlane *et al*, 2008) and *B. breve* (Djouzi *et al*, 1995) to utilize extensively galacto-oligosaccharides for growth was previously reported *in vivo*, whereas only a few *Lactobacillus* species could use this substrate efficiently, unlike *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 β-galactosidases, as evidenced for *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 *E. coli* and *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 *L. rhamnosus* enables a more significant reduction in pathogenic *C. perfringens* and an increase in health-promoting *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 *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 *C. perfringens* and *E. coli*, and positive correlations between SCFAs and bifidobacteria, lactobacilli and *Bacteroides distasonis* consistently indicate a link between SCFA production and certain changes in bacterial populations, such as reduction in pathogens. Moreover, *C. perfringens* being a primary butyrate producer, the specific anti-correlation with butyrate may also indicate that in response to stressors, *C. perfringens* activities may be shifted and stimulated. In particular, measures of bacterial activities in similar mouse models showed that the reduction of *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 TMA (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 glycogen (Kliewer *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 glycemia (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 normal delivery and breast-fed. A total of seven bacterial species were isolated, namely *E. coli*, *B. breve* and *B. longum*, *Staphylococcus epidermidis* and *S. aureus*, *C. perfringens* and *Bacteroides distasonis*, and they were mixed in equal amounts (approximately 10^{10} cells/ml for each strain) for gavage. Bacterial cell mixtures were kept in frozen aliquots until use. *L. paracasei* NCC2461 and *L. rhamnosus* NCC4007 probiotics were obtained from the Nestlé Culture Collection (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 10^8 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 Pre1 here. Pre1 comprises 75% dry matter in syrup and on a dry matter basis is composed of 23% lactose, 22% glucose, 0.8% galactose and 57% galactosyl-oligosaccharides with a degree of polymerization (DP) ranging between 3 and 9, and primarily composed of β-1,4 linkages. Amaretti *et al* (2007) previously reported the relative sugar composition of Vivinal GOS. DP 3 oligomer formed a major part of the oligosaccharides used and accounted for 37% of total carbohydrates, whereas the concentration of other oligomers decreased with the increase in DP (Amaretti *et al*, 2007). Two additional groups of HBM mice were fed with a diet containing 3 g per 100 g diet of an in-house preparation of galacto-oligosaccharides, 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 probiotic, 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 10^8 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. For jejunal microbiota analysis, the first 8 cm of the jejunum was collected into sterile tubes containing Ringer solution (Oxoid, UK), homogenized and kept on ice before microbial profiling. The cecal content was collected in Eppendorf™ tubes and a central section of the median lobe of the liver was dissected. Samples were snap-frozen immediately and kept at −80°C before analysis.

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. paracasei* 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 Drigalski medium (Bio-Rad, Switzerland), *Bacteroides distasonis* on Shaedler Neo Vanco medium (BioMérieux, Switzerland) and *S. aureus* and *S. epidermidis* 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. paracasei*, *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) $HgCl_2$ and 1% (v/v) H_3PO_4) 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-FFAP 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.

^{1}H 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% D_2O as the locking substance. Urine samples were prepared by mixing 20 μl of samples with 30 μl of a phosphate buffer solution containing 90% D_2O and 0.25 mM 3-trimethylsilyl-1-[2,2,3,3-$^2H_4$] propionate (TSP), which was used as

a chemical shift reference, into 1.7 mm NMR tubes. Fecal pellets were homogenized in 650 μl of a phosphate buffer solution containing 90% D_2O 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 10 min. Aliquots of 550 μl were then pipetted into 5 mm NMR tubes. Intact liver samples were bathed in an ice-cold saline D_2O solution. A portion of the tissue (~15 mg) was packed into a zirconium oxide 4 mm outer-diameter rotor.

All ^{1}H NMR spectra were recorded on a Bruker DRX 600 NMR spectrometer (Bruker Biospin, Rheinstetten, Germany) operating at 600.11 MHz for ^{1}H observation. ^{1}H NMR spectra of plasma, urine and fecal extracts were acquired with a Bruker 5 mm TXI triple-resonance probe at 298 K. ^{1}H 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 N_2 gas to minimize any time-dependent biochemical degradation.

^{1}H 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°-(τ-180°-τ-)*n*-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 ($2n\tau$) 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 COrrelation SpectroscopY (COSY) (Nagayama, 1980) and 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 ^{1}H observation equipped with a Bruker 5 mm SEI (^{1}H-^{13}C) 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 STOCSY on 1D spectra.

Multivariate statistical analysis and visualization

Statistical analysis of the changes in animal weights, bacterial populations and in the cecal composition of SCFAs obtained by GC was carried out using a two-tailed Mann–Whitney test.

The ^{1}H 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 only, 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 Q^2 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 Q^2 parameters obtained using random permutations are significantly different and lower than the experimental Q^2 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 (Rgraphsviz) 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 SCFA 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).

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