Probiotic modulation of symbiotic gut microbial–host metabolic interactions in a humanized microbiome mouse model

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The transgenomic metabolic effects of exposure to either Lactobacillus paracasei or Lactobacillus rhamnosus probiotics have been measured and mapped in humanized extended genome mice (germ-free mice colonized with human baby flora). Statistical analysis of the compartmental fluctuations in diverse metabolic compartments, including biofluids, tissue and cecal short-chain fatty acids (SCFAs) in relation to microbial population modulation generated a novel top-down systems biology view of the host response to probiotic intervention. Probiotic exposure exerted microbiome modification and resulted in altered hepatic lipid metabolism coupled with lowered plasma lipoprotein levels and apparent stimulated glycolysis. Probiotic treatments also altered a diverse range of pathways outcomes, including amino-acid metabolism, methylamines and SCFAs. The novel application of hierarchical-principal component analysis allowed visualization of multicompartmental transgenomic metabolic interactions that could also be resolved at the compartment and pathway level. These integrated system investigations demonstrate the potential of metabolic profiling as a top-down systems biology driver for investigating the mechanistic basis of probiotic action and the therapeutic surveillance of the gut microbial activity related to dietary supplementation of probiotics.

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Introduction

The gut microbiome–mammalian ‘Superorganism’ (Lederberg, 2000) represents a level of biological evolutionary development in which there is extensive ‘transgenomic’ modulation of metabolism and physiology that is a characteristic of true symbiosis. By definition, superorganisms contain multiple cell types, and the coevolved interacting genomes can only be effectively studied as an in vivo unit in situ using top-down systems biology approaches (Nicholson, 2006; Martin et al, 2007a). Interest in the impact of gut microbial activity on human health is expanding rapidly and many mammalian–microbial associations, both positive and negative, have been reported (Dunne, 2001; Verdu et al, 2004; Nicholson et al, 2005; Gill et al, 2006; Ley et al, 2006). Mammalian–microbial symbiosis can play a strong role in the metabolism of endogenous and exogenous compounds and can also be influential in the etiology and development of several diseases, for example insulin resistance (Dumas et al, 2006), Crohn’s disease (Gupta et al, 2000; Marchesi et al, 2007), irritable bowel syndrome (Sartor, 2004; Martin et al, 2006), food allergies (Bjorksten et al, 2001), gastritis and peptic ulcers (Warren, 2000; Marshall, 2003), obesity (Ley et al, 2006; Turnbaugh et al, 2006), cardiovascular disease (Pereira and Gibson, 2002) and gastrointestinal cancers (Dunne, 2001). Activities of the diverse gut microbiota can be highly specific and it has been reported that the establishment of Bifidobacteria is important for the development of the immune system and
for maintaining gut function (Blum and Schiffrin, 2003; Salminen et al., 2005; Ouwehand, 2007). In particular, elevated counts in *Bifidobacterium* with reduced *Escherichia coli*, streptococci, *Bacteroides* and clostridia counts in breast-fed babies compared to formula-fed neonates may result in the lower incidence of infections, morbidity and mortality in breast-fed infants (Dai et al., 2000; Kunz et al., 2000). As the microbiome interacts strongly with the host to determine the metabolic phenotype (Holmes and Nicholson, 2005; Gavaghan McKee et al., 2006) and metabolic phenotype influences outcomes of drug interventions (Nicholson et al., 2004; Clayton et al., 2006), there is clearly an important role of understanding these interactions as part of personalized healthcare solutions (Nicholson, 2006).

One of the current approaches used to modulate the balance of intestinal microflora is based on oral administration of probiotics. A probiotic is generally defined as a 'live microbial feed supplement which beneficially affects the host animal by improving its intestinal microbial balance' (Fuller, 2004). The gastrointestinal system is populated by potentially pathogenic bacteria that are capable of degrading proteins (putrefaction), releasing ammonia, amines and indoles, which in high concentrations can be toxic to humans (Cummings and Bingham, 1987). Probiotic supplementation aims at replacing or reducing the number of potentially harmful *E. coli* and *Clostridia* in the intestine by enriching the populations of gut microbiota that ferment carbohydrates and that have little proteolytic activity. Probiotics, most commonly *Lactobacillus* and *Bifidobacteria*, can be used to modulate the balance of the intestinal microflora in a beneficial way (Collins and Gibson, 1999). Although Lactobacilli do not predominate among the intestinal microflora, their resistance to acid conditions and bile salts toxicity results in their ubiquitous presence throughout the gut (Corcoran et al., 2005), hence they can exert metabolic effects at many levels. Fermented dairy products containing *Lactobacillus* have traditionally been used to modulate the microbial ecology (Dunne, 2001). In particular, *L. paracasei* was shown to modulate the intestinal physiology, to prevent infection of pathogenic bacteria (Sarker et al., 2005), to stimulate the immune system (Ihnow-Zekri et al., 2003), and to normalize gastrointestinal disorders (Martin et al., 2006). *L. rhamnosus* is also a significant probiotic strain with proven health benefits and therapeutic applications in the treatment of diarrhea (Szyanski et al., 2006), irritable bowel syndrome (Kajander et al., 2005), atopic eczema (Corcoran et al., 2005) and the prevention of urinary tract infections (Reid and Bruce, 2006). However, the functional effects of probiotic interventions cannot be fully assessed without probing the biochemistry of the host at multiple compartmental levels, and we propose that top-down systems biology provides an ideal approach to further understanding in this field. The microbiota observed in human baby flora (HBF) mice have a number of similarities with that found in formula-fed neonates (Mackie et al., 1999), which makes it to be a well-adapted and simplified model to assess probiotics impact on gut microbial functional ecosystems (in particular on metabolism of *Bifidobacteria* and potential pathogens) and subsequent effects on host metabolism.

Metabolic profiling using high-density data generating spectroscopic techniques, in combination with multivariate mathematical modelling is a tool which is well suited to generate metabolic profiles that encapsulate the top-down system response of an organism to a stressor or intervention (Nicholson and Wilson, 2003). Multivariate metabolic profiling offers a practical approach to measuring the metabolic endpoints that link directly to whole system activity and which are determined by both host genetic and environmental factors (Nicholson et al., 2005). Recently, metabolic profiling strategies have been successfully applied to characterizing the metabolic consequences of nutritional intervention (Rezzi et al., 2007; Wang et al., 2007) the effects of the gut microflora on mammalian metabolism (Martin et al., 2006, 2007a, b) and mechanisms of insulin-resistance (Dumas et al., 2006). In the current study, 1H nuclear magnetic resonance (NMR) spectroscopy and targeted ultra performance liquid chromatography-mass spectrometry (UPLC-MS) analysis have been applied to characterize the global metabolic responses of humanized microbiome mice subsequently exposed to placebo, *Lactobacillus paracasei* or *Lactobacillus rhamnosus* supplementation. Correlation of the response across multiple biofluids and tissue, using plasma, urine, fecal extracts, liver tissues and ileal flushes as the biological matrices for the detection of dietary intervention, generates a top-down systems biology view of the response to probiotics intervention.

**Results**

**Gut bacterial composition**

Microbiological analyses were performed on fecal samples to assess the growth of the HBF in germ-free mice and to ascertain the effects of probiotics on the development of gut bacteria. The measured terminal composition of the fecal microbiota is detailed in Table I, where the statistically significant differences between the various groups were calculated using a two-tailed Mann–Whitney test. The bacterial populations of *Bifidobacteria longum* and *Staphylococcus aureus* were reduced after introduction of both probiotics. Additionally, unique effects of *L. rhamnosus* supplementation caused decreased populations of *Bifidobacterium breve*, *Staphylococcus epidermidis* and *Clostridium perfringens* but an increase of *E. coli*.

**Gut levels of short-chain fatty acids**

Short-chain fatty acids (SCFAs), namely acetate, propionate, isobutyrate, n-butyrate and isovalerate, were identified and quantified from the cecal content using GC-FID. The results, presented in Table II, are given in µmol per gram of dry fecal material and as mean ± s.d. for each group of mice. The production of some of the SCFAs, that is, acetate and butyrate, by the HBF mice supplemented with both of the probiotics was reduced. In addition, increases of the concentrations in isobutyrate and isovalerate were observed in the mice fed with *L. paracasei*.

**Analysis of 1H NMR spectroscopic data on plasma, urine, liver and fecal extracts**

A series of pairwise O-PLS-DA models of 1H NMR spectra were performed to extract information on the metabolic effects of
Table I  Microbial species counts in mouse feces at the end of the experiment

| Groups/log10 CFU | HBF (n = 10) | HBF + L. paracasei (n = 9) | HBF + L. rhamnosus (n = 9) |
|-----------------|-------------|---------------------------|---------------------------|
| L. paracasei    | —           | 8.5 ± 0.2                 | —                         |
| L. rhamnosus    | —           | 9.4 ± 0.3                 | 7.8 ± 0.2                 |
| E. coli         | 9.2 ± 0.3   | 9.4 ± 0.3                 | 9.8 ± 0.5**               |
| B. breve        | 9.1 ± 0.2   | 7.78 ± 2.13               | 8.7 ± 0.3*                |
| B. longum       | 8.2 ± 0.6   | 5.6 ± 1.9***              | 6.3 ± 0.5***              |
| S. aureus       | 7.4 ± 0.3   | 6.3 ± 0.3***              | 6.6 ± 0.5***              |
| S. epidermidis  | 4.8 ± 0.4   | 4.9 ± 1.2                 | 4.0 ± 0.5***              |
| C. perfringens  | 7.2 ± 0.3   | 7.0 ± 0.5                 | 5.7 ± 1.0***              |
| Bacteroides     | 10.3 ± 0.2  | 10.4 ± 0.2                | 10.1 ± 0.4                |

log10 CFU (colony-forming unit) given per gram of wet weight of feces. Data are presented as mean ± s.d. Absence of specific bacterial strains in the gut microflora is indicated by “—”. The values for the HBF mice supplemented with probiotics were compared to HBF control mice, */ ** and *** indicate a significant difference at 95, 99 and 99.9% confidence levels, respectively.

Table II  Short-chain fatty acid content in the cecum from the different groups

| Amounts of SCFAs given in µmol per gram of dry feces for each group | Acetate | Propionate | Isobutyrate | Butyrate | Isovalerate |
|---------------------------------------------------------------------|--------|------------|-------------|----------|------------|
| HBF (n = 10)                                                        | 77.6 ± 17.6 | 22.3 ± 4.3 | 0.9 ± 0.2  | 3 ± 0.6  | 2.1 ± 0.6  |
| HBF + L. paracasei (n = 9)                                          | 52.3 ± 23.6*** | 22.2 ± 10.8 | 1.2 ± 0.5*** | 1.5 ± 0.8*** | 2.7 ± 1.2** |
| HBF + L. rhamnosus (n = 9)                                          | 40.6 ± 8*** | 20.3 ± 2.8  | 0.8 ± 0.2  | 2.1 ± 0.4*** | 2.1 ± 0.5  |

Data are presented in µmol per gram of dry feces and are presented as means ± s.d. The amounts of SCFAs for the HBF mice supplemented with probiotics were compared to HBF control mice, */ ** and *** indicate a significant difference at 99 and 99.9% confidence levels, respectively.

probiotic modulation. A statistically significant metabolic phenotype separation between untreated mice and probiotic supplemented animals was observed as reflected by the high value of Q5 for each model (Cloarec et al., 2005b; Table III). The corresponding coefficients describing the most important metabolites in plasma, liver, urine and fecal extracts that contributed to group separation are also listed in Supplementary Table I. The area normalized intensities (10^3 a.u.) of representative metabolite signals are given as means ± s.d. in Table III. The O-PLS-DA coefficients plots are presented in Figure 1 using a back-scaling transformation and projection to aid biomarker visualization (Cloarec et al., 2005b). The direction of the signals in the plots relative to zero indicates positive or negative covariance with the probiotic-treated class. Each variable is plotted with a color code that indicates its discriminating power as calculated from the correlation matrix thus highlighting biomarker-rich spectral regions.

Liver metabolic profiles
Livers of mice fed with L. paracasei showed relative decreases in dimethylamine (DMA), trimethylamine (TMA), leucine, isoleucine, glutamine, and glycogen and increased levels of succinate and lactate (Figure 1A). Mice supplemented with L. rhamnosus showed relative decreases in leucine and isoleucine and relative increases in succinate, TMA and trimethylamine-N-oxide (TMAO) in the liver compared to controls (Figure 1D).

Plasma metabolic profiles
Plasma samples showed relative decreases in the levels of lipoproteins and increases in the concentrations of glycerophosphorylcholine (GPC) and triglycerides in mice fed with both probiotics compared to controls (Figure 1B and E). Elevated choline levels were observed in plasma of mice fed with L. rhamnosus and reduced plasma citrate levels were observed in mice fed with L. paracasei compared to controls.

Fecal extract metabolic profiles
Marked changes were observed in the metabolic profiles of fecal extracts from all supplemented mice, for example relative decreased concentrations of choline, acetate, ethanol, a range of putative N-acetylated metabolites (NAMs), unconjugated bile acids (BAs) and tauro-conjugated bile acids (Figure 1C and F). Furthermore, relative higher levels of glucose, lysine and polysaccharides were detected in the feces from mice fed with probiotics. A relative increased level of n-caproate (chemical shifts δ at 0.89(t), 1.27(m), 1.63(q), 2.34(t)) appeared to be associated with mice supplemented with L. paracasei.

Urine metabolic profiles
Urine samples of mice supplemented with both probiotics showed relative increased concentrations of indoleacetylglucine (IAG), phenylacetylglutamine (PAG), tryptamine and a relative decrease in the levels of N-keto-isocaproate and citrate (Figure 1G and H). Relative increased concentrations of a mixture of putative glycolipids (UGLp, chemical shifts of multiplets at δ 0.89, 1.27, 1.56, 1.68, 2.15, 2.25, 3.10, 3.55, 3.60), N-acetyl-glycoproteins (NAGs) and a reduction in 3-hydroxy-isovalerate were also observed in mice supplemented with L. paracasei compared to controls. Urine of mice fed with L. rhamnosus showed a reduction in levels of creatine and citrulline.
UPLC-MS analysis of bile acids in ileal flushes

The proportion of bile acids in ileal flushes from the different groups are given in Table IV and are shown as mean ± s.d. of the percentage of the total bile acid content. O-PLS-DA of the data set revealed that the relative concentrations of bile acids obtained from unsupplemented HBF mice are separated from those treated with probiotics, the correlation observed with L. paracasei being more significant than with L. rhamnosus as noted by the values of the cross-validated model parameter $Q^2$ (Figure 2). For example, HBF mice supplemented with L. paracasei showed strong correlations with higher amounts of GCA, CDCA and UDCA and lower levels of α-MCA in the ileal flushes when compared to controls. HBF mice fed with L. rhamnosus also showed higher levels of GCA associated with lower levels of TUDCA and TDCA in the ileal flushes when compared to untreated HBF mice.

Integration of multicompartment metabolic data using hierarchical-principal component analysis

A principal component analysis (PCA) model was initially constructed separately for the metabolic data from each individual biological matrix (plasma, urine, liver, fecal extracts and bile acid composition in ileal flush; Figures 3 and 4). Three principal components were calculated for each cross-validated PCA model, except for the plasma where four principal components were calculated for each cross-validated model. The score vectors $t_y$ from each model were then assigned as new X-variables (Figure 3), except for the plasma where four principal components were calculated for each cross-validated model. The score vectors $t_y$ from each model were then assigned as new X-variables (Figure 3), except for the plasma where four principal components were calculated for each cross-validated model. The score vectors $t_y$ from each model were then assigned as new X-variables (Figure 3), except for the plasma where four principal components were calculated for each cross-validated model.

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

| Metabolites   | Chemical shift and multiplicity | HBF controls | HBF + L. paracasei | HBF + L. rhamnosus |
|--------------|---------------------------------|--------------|--------------------|--------------------|
| Liver        |                                 |              |                    |                    |
| Leu          | 0.92(t)                         | 2.4 ± 0.6    | 1.7 ± 0.3***       | 1.9 ± 0.5*         |
| Ileu         | 0.94(t)                         | 0.8 ± 0.1    | 0.6 ± 0.05***      | 0.7 ± 0.2          |
| Lactate      | 1.32(d)                         | 38.4 ± 5.8   | 46.2 ± 8.2*        | 39.2 ± 9.7         |
| Succinate    | 2.41(s)                         | 0.2 ± 0.1    | 1.0 ± 0.6**        | 0.7 ± 0.3*         |
| MA           | 2.61(s)                         | 0.1 ± 0.06   | 0.04 ± 0.002**     | 0.08 ± 0.05        |
| TMA          | 2.91(s)                         | 0.2 ± 0.04   | 0.07 ± 0.03***     | 0.25 ± 0.09        |
| TMAO         | 3.27(s)                         | 10.3 ± 2.2   | 13.1 ± 3.7         | 18.5 ± 8.0**       |
| Glu          | 2.44(m)                         | 0.4 ± 0.1    | 0.3 ± 0.1*         | 0.3 ± 0.1          |
| Glycogen     | 5.38–5.45                       | 3.4 ± 1.9    | 1.5 ± 0.6*         | 3.2 ± 1.9          |
| Plasma       |                                 |              |                    |                    |
| Lipoproteins | 0.84(m)                         | 13.7 ± 1.8   | 10.1 ± 0.9***      | 9.8 ± 4.2**        |
| Citrate      | 2.65(d)                         | 1.4 ± 0.3    | 0.9 ± 0.4**        | 1.1 ± 0.2**        |
| Choline      | 3.2(s)                          | 11.6 ± 2.6   | 16.2 ± 5.7*        | 20.5 ± 3.8**       |
| GPC          | 3.22(5)                         | 44.1 ± 4.6   | 57.3 ± 12.5***     | 68.1 ± 11.2***     |
| Glyceryls    | 3.91(m)                         | 2.0 ± 0.3    | 2.5 ± 0.4**        | 2.7 ± 0.4**        |
| Feces        |                                 |              |                    |                    |
| Caprylate    | 1.27(m)                         | 2.5 ± 0.1    | 3.5 ± 0.2**        | 2.4 ± 0.1          |
| Lys          | 3.00(m)                         | 3.2 ± 0.8    | 5.0 ± 0.3***       | 4.0 ± 1.2**        |
| Oside        | 5.42(m)                         | 0.9 ± 0.09   | 1.2 ± 0.1***       | 1.4 ± 0.1***       |
| Bile acids   | 0.72(s)                         | 3.1 ± 0.9    | 1.8 ± 0.7**        | 2.0 ± 0.6*         |
| Ethanol      | 1.18(t)                         | 2.5 ± 0.1    | 2.0 ± 0.08***      | 1.9 ± 0.09***      |
| Choline      | 3.20(s)                         | 48.0 ± 19.5  | 11.3 ± 4.1***      | 20.1 ± 10.9**      |
| NAM          | 2.06(m)                         | 7.1 ± 1.0    | 5.4 ± 0.3***       | 5.5 ± 0.3***       |
| Acetate      | 1.91(s)                         | 58.7 ± 34.2  | 27.0 ± 9.2***      | 32.9 ± 12.9*       |
| U1           | 3.71(s)                         | 9.2 ± 0.5    | 7.3 ± 0.3***       | 8.2 ± 0.5**        |
| Urine        |                                 |              |                    |                    |
| IAG          | 7.55(d)                         | 0.1 ± 0.03   | 0.6 ± 0.2***       | 0.4 ± 0.2**        |
| PAG          | 7.37(m)                         | 0.8 ± 0.1    | 1.5 ± 0.3***       | 1.2 ± 0.4*         |
| Tryptamine   | 7.70(d)                         | 0.1 ± 0.04   | 0.4 ± 0.1***       | 0.2 ± 0.1**        |
| UGLp         | 1.27(m)                         | 1.7 ± 0.1    | 2.7 ± 0.4**        | 1.7 ± 0.2          |
| Glycero-metabolites | 4.04 (m) | 1.7 ± 0.1 | 2.2 ± 0.2*** | 1.9 ± 0.2* |
| NAG          | 2.04(s)                         | 3.5 ± 0.2    | 4.3 ± 0.2***       | 3.8 ± 0.5          |
| Butyrate     | 0.90(t)                         | 6.9 ± 0.8    | 5.2 ± 0.7**        | 5.2 ± 0.9**        |
| α-keto-isocaproate | 0.94(d) | 13.8 ± 4.6 | 6.1 ± 2.3** | 7.9 ± 2.1** |
| Propionate   | 1.05(t)                         | 0.9 ± 0.2    | 0.8 ± 0.04*        | 0.8 ± 0.1          |
| 3-hydroxy-isovalerate | 1.24(s) | 3.0 ± 0.4 | 2.1 ± 0.4** | 2.6 ± 0.2 |
| Citrate      | 2.55(d)                         | 10.8 ± 7.6   | 1.6 ± 0.6**        | 2.2 ± 0.9*         |
| Creatine     | 3.92(s)                         | 5.7 ± 2.1    | 4.5 ± 1.5          | 3.5 ± 0.3**        |
| Citrulline   | 1.88(m)                         | 3.8 ± 0.5    | 3.3 ± 0.4          | 3.0 ± 0.4**        |

O-PLS models were generated for comparing probiotics treated to HBF control mice using one predictive and two orthogonal components. $R^2$ value shows how much of the variation is explained. $Q^2$ value represents the predictability of the models and relates to its statistical validity. Data are presented as area normalized intensities (10³ a.u.) of representative metabolite signals as means ± s.d. The values for the HBF mice supplemented with probiotics were compared to HBF control mice. * * * indicate a significant difference at 90, 95, 99 and 99.9% confidence levels, respectively.

$R^2 = 90%$, $R^2 = 48%$

$R^2 = 89%$, $R^2 = 49%$

$R^2 = 91%$, $R^2 = 50%$

$R^2 = 44%$, $R^2 = 44%$

$R^2 = 41%$, $R^2 = 37%$

$Q^2 = 41%$, $R^2 = 32%$

$Q^2 = 51%$, $R^2 = 32%$

$Q^2 = 44%$, $R^2 = 44%$

$Q^2 = 49%$, $R^2 = 49%$

$Q^2 = 59%$, $R^2 = 46%$

$Q^2 = 59%$, $R^2 = 46%$

$Q^2 = 41%$, $R^2 = 44%$

$Q^2 = 51%$, $R^2 = 32%$
Pi (plasma PCs 1–4), Li (liver PCs 1–3), Ui (urine PCs 1–3), Bi (bile acid PCs 1–3) and Fi (fecal PCs 1–3), which comprise only the systematic variation from each of the blocks/compartments. The two first principal components (p1 and p2) calculated for the hierarchical-principal component analysis (H-PCA; Westerhuis et al., 1998) model ($R^2_X=0.60$) accounted for 37 and 23% of the total variance in the combined multi-compartment data respectively. The cross validation for the H-PCA model failed due to the high degree of orthogonality within the X-matrix, that is, within each of the blocks all variables

Figure 1  O-PLS-DA coefficient plots derived from $^1$H MAS NMR CPMG spectra of liver (A, D), $^1$H NMR CPMG spectra of plasma (B, E), $^1$H NMR standard spectra of fecal extracts (C, F) and urine (G, H), indicating discrimination between HBF mice fed with probiotics (positive) and HBF control mice (negative). The color code corresponds to the correlation coefficients of the variables with the classes. BAs, Bile acids; DMA, dimethylamine; Glc, glucose; Gin, glutamine; GPC, glycerophosphorylcholine; IAG, indoleacetylglycine; Ileu, isoleucine; Leu, leucine; Lys, lysine; NAG, N-acetylated glycoproteins; NAM, N-acetylated metabolites; Osides, glycosides; PAG, phenylacetylglycine; TBAs, taurine conjugated to bile acids; TMA, trimethylamine; TMAO, trimethylamine-N-oxide; UGLp, unidentified glycolipids.
are orthogonal to each other, while each of the biological matrices could be cross-validated at the individual level.

The H-PCA scores plot illustrated a degree of clustering with respect to the groups of HBF mice (Figure 4A). The corresponding H-PCA loadings plot indicated the contribution of the 16 descriptors to the differences observed between the samples in the H-PCA scores plot. Probiotic-supplemented HBF animals are separated from the controls along the first principal component, and this arises from the main variations modelled at the base level PCA of the individual plasma (P1, P2), liver (L1, L2), ileal flush (B2) and urine (U1, U2) data sets (Figure 4B). HBF mice fed with L. paracasei were separated from those fed with L. rhamnosus along the second principal component, which was mainly due to the variations modelled at the base level PCA of plasma (P2, P3), liver (L1) and urine (U1, U3). Interestingly, the metabolic variations in the fecal samples have no weight in discriminating the bacterial supplementation from the corresponding controls in the global model.

To uncover variables contributing to the H-PCA super scores, the loadings at the base level PCA model were interrogated (Figure 4C–F). HBF mice supplemented with probiotics showed higher concentrations of glucose, choline, GPC, glutamine, glutamate and lysine in the plasma profiles associated with elevated concentrations of glucose in the liver and higher levels of TUDCA and TCDCA in the ileal flushes. Controls showed higher levels of lipoproteins in the plasma, elevated concentrations of lipids, glycogen, glutamine, glutamate, alanine, TMAO and lactate in the liver, associated with higher levels of TCA and TβMCA in the ileal content. Unsupplemented HBF mice also showed elevated urinary excretions of creatine, citrate, citrulline, lysine, UGLp, NAG and α-keto-isocaproate compared to animals fed with probiotics. Moreover, H-PCA also revealed that HBF mice treated with L. rhamnosus had higher levels of hepatic lipids and plasma lipoproteins but lower concentrations of lactate and amino acids in plasma and lower urinary excretion of PAG, IAG, taurine and taurine than HBF mice treated with L. paracasei.

Table IV Bile acids composition in gut flushes for the different microbiota

| Microbiota/Bile acids | Microbiota/Bile acids | Microbiota/Bile acids |
|-----------------------|-----------------------|-----------------------|
|                       | HBF                   | HBF + L. paracasei    | HBF + L. rhamnosus |
| CDCA                  | ND                    | 0.04 ± 0.07           | 0.01 ± 0.02        |
| UDCA                  | ND                    | 0.1 ± 0.1             | ND                  |
| CA                    | 0.4 ± 0.5             | 0.3 ± 0.7             | 0.3 ± 0.2           |
| αCA                   | ND                    | 0.02 ± 0.07           | ND                  |
| βCA                   | 0.3 ± 0.2             | 0.3 ± 0.6             | 0.2 ± 0.2           |
| γCA                   | 0.9 ± 0.7             | 1.3 ± 2.7             | 0.8 ± 0.7           |
| GCA                   | ND                    | 0.1 ± 0.1             | ND                  |
| TDCDA                 | 3.3 ± 1               | 4 ± 2.1               | 2.9 ± 1.3           |
| TUDCA                 | 6.6 ± 1.4             | 6.6 ± 5.2             | 5.1 ± 1.02          |
| TβMCA                 | 50 ± 4.8              | 49.2 ± 7.5            | 50.5 ± 5.2          |
| TCA                   | 38 ± 3.5              | 38 ± 6.2              | 40.4 ± 5            |

Relative composition in bile acids given in percentage of total bile acid content. Species not detected with UPLC-MS experiment are shown as ND. The key is given in UPLC-MS material and methods.

Integration of correlations between bile acids and fecal flora

We further investigated the connections between fecal flora and intestinal bile acids using a correlation analysis based bipartite graphical modelling approach (see Materials and methods; Figure 5) used previously to investigate the effects of gut microbiome humanization in germfree mice (Martin et al., 2007a). In the current study, we are working with a superior...
Liver Pyruvate Ala

contribution to the H-PCA model in red (principal component 1) and in blue (principal component 2). The model has been calculated from Pareto scaled data using two PCs 1–3), which can be combined to form a new data matrix than can then be modelled using PCA. The individual PCA loadings were color coded according to their

using PCA, which generates the 'super scores'

Figure 3  Schematic overview of H-PCA modelling; (Gunnarsson et al, 2003). In the sublevel, each block of data X, is modelled locally by a PCA model. Each block is summarized by one or more loading vectors p, and score vectors t, ('super variables'), which can be combined to form a new data matrix than can then be modelled using PCA, which generates the 'super scores' t and the 'super loadings' p. All conventional PCA statistics and diagnostics are retained.

Figure 4  H-PCA scores (A) and loadings (B) plots for the two first components derived from scores of separate PCA constructed separately for the metabolic data from each individual biological matrix from HBF mice (■), HBF-L. paracasei mice (●) and HBF-L. rhamnosus mice (○). These PCA models explained 94% (bile acid, C), 70% (plasma, D), 80% (liver, E), 41% (urine, F) and 61% (feces, data not shown) of the total variation in the data, respectively. The systematic variation from each of the block/compartment is summarized by its score vectors denoted P (plasma PCs 1–4), L (liver PCs 1–3), U (urine PCs 1–3), B ( bile acid PCs 1–3) and F (fecal PCs 1–3), which can be combined to form a new data matrix than can then be modelled using PCA. The individual PCA loadings were color coded according to their contribution to the H-PCA model in red (principal component 1) and in blue (principal component 2). The model has been calculated from Pareto scaled data using two cross-validated PCs, R²X=80%. Ala, alanine; see Figure 1.

model where all the major bacteria strains are identified, which was not possible when considering conventional microflora. Positive and negative correlations show the multicolinearity between bile acids and gut bacteria, whose concentrations are interdependent such as in the case of substrate–product biochemical reactions. Additional pixel maps of the correlation matrices are given to help interpretation in Supplementary Figure 1.
Control HBF mice and HBF mice supplemented with probiotics show remarkably different bile acid/fecal flora correlation networks (Figure 5A–C), indicating that small modulations in the species composition of the microbiome can result in major functional ecological consequences. Network analysis for HBF mice supplemented with L. paracasei reveals that Lactobacilli supplementation resulted in decreasing the functional links between Bifidobacteria and bile acids, while new significant correlations were observed between bile acids, and Bacteroides, S. aureus, S. epidermidis and L. paracasei. Moreover, E. coli has several connections with UDCA, aMCA and TCDCA. Interestingly, Bacteroides shows functional correlations of opposite signs for TbMCA, TUDCA and TCA when compared to Lactobacilli and Staphylococci.

When HBF mice received L. rhamnosus probiotic, the microbiome/metabolome network shows a significant lower level of complexity (given for the cut-off value of 0.5). The balance within B. breve, S. aureus and S. epidermidis appears highly correlated to the composition in tauro-conjugated bile acids (TjMCA, TCA) and unconjugated bile acids (βMCA, CA).

Network analysis for HBF mice supplemented with L. paracasei reveals that Lactobacilli supplementation resulted in decreasing the functional links between Bifidobacteria and bile acids, while new significant correlations were observed between bile acids, and Bacteroides, S. aureus, S. epidermidis and L. paracasei. Moreover, E. coli has several connections with UDCA, aMCA and TCDCA. Interestingly, Bacteroides shows functional correlations of opposite signs for TjMCA, TUDCA and TCA when compared to Lactobacilli and Staphylococci.
Discussion

The cometabolic processes regulating mammalian systems and their coexisting gut microbiota are an essential evolutionary driver towards providing more refined control mechanisms on the host physiology (Pereira and Gibson, 2002; Pereira et al., 2003; Backhed et al., 2004; Holmes and Nicholson, 2005; Nicholson et al., 2005; Dumas et al., 2006; Martin et al., 2006, 2007a). In the present study, we demonstrate a significant association between the probiotic modulation of the gut microbiome and the metabolism of SCFAs, amino acids and methylamines, bile acids and plasma lipoproteins, and also an association with stimulated glycolysis, showing the remarkable diversity of symbiotic cometabolic connections.

Gut microbiotal links to host energy metabolism

We have recently described that HBF mice supplemented with _L. paracasei_ were characterized by a high gut content of tauro-conjugated bile acids due to the inability of gut flora to deconjugate the bile acids, which resulted in high intestinal conjugated bile acids due to the inability of gut flora to deconjugate the hepatic tauro-conjugated bile acids (Midtvedt and Norman, 1967; Floch, 2002). Moreover, the main source of dietary lipids in animal chow is soybean oil, which is composed at 65% of long-chain polyunsaturated fatty acids. It is well known that _Lactobacillus_ hydrolyzes soy oil to conjugated linoleic acid efficiently (Xu et al., 2005), which results in a reduction of plasma lipoprotein concentrations and hepatic cholesterol (Fukushima et al., 1996; Al-Othman, 2000) and in the inhibition of _S. aureus_ growth (Das, 2002), as observed in the current study. Furthermore, probiotics supplementation was associated with significant reduction of acetate in the cecal content (Table II) and in a reduced hepatic acetate:propionate ratio, for which a serum lipids lowering effect has previously been described (Wong et al., 2006). Our results illustrate the fine relationship between a specific gut microbial population modulation and the host’s lipid metabolism and that a probiotic intervention can provide refined control mechanisms on the host’s physiology.

Furthermore, the molecular foundations of beneficial symbiotic host–bacteria relationships lie in the critical involvement of the microbiome in calorie recovery through further processing of dietary nutrients and indigestible fibers. Levels of leucine and isoleucine were reduced together with their keto-acid derivative (z-keto-isocaproate) in _L. paracasei_-supplemented mice. These observations suggest higher catabolism of branched-chain amino acids to produce acetyl-CoA and glucose via gluconeogenesis. Decreased levels of citrate in urine and plasma, but increased liver succinate levels may also indicate the shunt of the tricarboxylic acid cycle towards production of phosphoenolpyruvate for gluconeogenesis in _L. paracasei_-supplemented mice. Moreover, reduction of liver glycogen observed with _L. paracasei_ supplementation is consistent with our other observations of generalized host mobilization of other metabolic fuels.

Probiotics induce specific microbiome–host transgenicomic metabolic interactions

We investigated the relationship between probiotic-induced changes in gut microbes and bile acid cometabolism using bipartite graphs to display correlation patterns between fecal flora and bile acids (Figure 5). Correlation analysis derived from bile acid and fecal flora profiles offers a unique approach to capture subtle variations in bile acid composition that may be directly related to changes in gut microbial levels, and that may be induced by accumulation of bile acids in _Lactobacillus_ probiotics for instance. Control HBF mice and HBF mice supplemented with probiotics show remarkably different bile acid/fecal flora correlation networks. The different bacterial strains of Bacteroides, Clostridia, Streptococci and Lactobacilli share similar abilities to deconjugate the hepatic tauro-conjugated bile acids (Midveldt and Norman, 1967; Floch, 2002). In that regard, the overwhelming contrast between the balance of these bacteria on one hand, and conjugated bile acids (TCDDCA, TβDCCA, TCA, TUDCA) and unconjugated bile acids (CA, αMCA, βMCA) on the other hand, highlights the metabolic flexibility of the gut microbiota in response to probiotics supplementation. These different correlative patterns further characterize the microbiob–mammalian transgenicomic metabolic interactions, whereby probiotics-induced modulation of the gut microbial functional ecosystem results in different bile acid composition (Figure 2) and enterohepatic recirculation.

The relationship between specific gut microbial strains and bile acid cometabolism is well illustrated with the contrast between _Lactobacillus_, which shows resistance to bile salt toxicity (Corcoran et al., 2005), and _C. perfringens_, which is sensitive to the strong growth inhibitory effects of unconjugated bile acids and TCDDCA (Kishinaka et al., 1994; Floch, 2002). In the absence of _Lactobacillus_ supplementation, _C. perfringens_ has anticorrelated connections with TCDDCA, αMCA and βMCA, which highlights the strong inhibitory effects of these bile acids on _C. perfringens_ growth. Interestingly, the probiotic supplementation was either associated with maintenance or decrease of the Clostridial population (Table I), while no functional correlations between _C. perfringens_ and bile acids were observed. In that regard, these observations may indicate different nutritional competition leading to modulation of _C. perfringens_ population and maintenance of the intestinal ecology. Consequently, interbacterial cooperation to transform bile acids is an important
factor that needs to be considered not only for the fine tuning of microbial balance but also to modulate dietary fat emulsification and absorption.

**Gut-bacterial production of methylamines via choline metabolism**

The elevation of methylamines (TMA, TMAO) in liver, choline and GPC in the plasma and decreased choline in feces from HBF mice supplemented with *L. rhamnosus* (Figure 1) are additional illustrations of the complex 'microbial–mammalian metabolic axis', as the microbiota are involved in the synthesis and metabolism of these methylamines (al-Waiz et al., 1992). The first reaction of the methylamine pathway involves conversion of dietary choline into TMA by gut microbiota (Zeisel et al., 1983), which is then detoxified to TMAO in the liver via the flavine mono-oxygenase system (Smith et al., 1994; Figure 6). *L. rhamnosus* supplementation contributes to higher absorption of free choline and may induce elevated production of methylamines by *Bacteroides* and *C. perfringens* (Allison and Macfarlane, 1989) through nutritional competition.

Interestingly, *L. paracasei* consumption may favor a different metabolic fate for choline through different bacterial reprocessing. Decreased fecal choline was associated with reduced concentrations of TMA and DMA in the liver, an increase in plasma GPC, but with no changes in liver TMAO and plasma choline after *L. paracasei* supplementation. These animals also showed a greater reduction in plasma lipoproteins when compared to other groups. Here, these observations may result from elevated bacterial consumption for cholesterol assimilation (Rasic, 1992) and phospholipid metabolism (Jenkins and Courtney, 2003; Taranto et al., 2003; Kankaanpaa et al., 2004). Thus, the reduced availability of choline to other bacterial strains may have led to lower production of methylamines and absorption of free choline into host metabolism.

**Probiotic modulation of amino-acid metabolism**

Investigation of the urine metabolic profiles showed significant increases in the concentrations of microbial comatabolites PAG, IAG and tryptamine in probiotic supplemented mice (Goodwin et al., 1994; Smith and Macfarlane, 1996). These metabolites are produced from amino acids, which after

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![Figure 6](image-url)  
*Figure 6* Gut-microbiota–mammalian cometabolism of methylamines and aromatic amino acids. DMG, dimethylglycine; IAA, indoleacetate; MA, methylamine; PA, phenylacetate (see Figure 1).
depolymerization of dietary proteins (casein) by pancreatic endopeptidases and bacterial proteases and peptidases, become available for fermentation by the gut microflora (Smith and Macfarlane, 1996), as outlined in Figure 6. Metabolism of the aromatic amino acids phenylalanine, tyrosine and tryptophan generates phenylacetate (PA), p-cresol, indoleacetate (IA) and tryptamine, respectively (Donaldson, 1962; Smith and Macfarlane, 1996). PA and IA may be detoxified in the gut mucosa and the liver by glycine conjugation forming PAG and IAG prior to excretion via the urine (Donaldson, 1962; Smith and Macfarlane, 1996). The production of PA and IA has been restricted to a certain taxonomic group of gut bacteria, including Bacteroides, Clostridia and E. coli, which count among the dominant species in HBF colonized mice (Smith and Macfarlane, 1996; Xu et al., 2002). The increased urinary excretion of phenolic and indolic compounds reflects variations in gut microflora composition in relation to nutritional competition (Smith and Macfarlane, 1996; Nowak and Libudzisz, 2006). For instance, IA has been reported to inhibit the growth and survival of Lactobacilli, and specifically L. paracasei (Nowak and Libudzisz, 2006).

In addition, increased levels of lysine in feces from mice supplemented with probiotics is another example of bacterial contribution to the mammalian amino-acid homeostasis (Metges, 2000) and hepatic protein synthesis (Metges et al., 2006; Figure 1). Higher production of bacterial isobutyrate and isovalerate (Table II) suggests increased bacterial fermentation of leucine and valine that can also influence host energy metabolism (Macfarlane et al., 1992). Moreover, L. paracasei supplementation specifically induced higher urinary excretion of NAG and decreased N-acetylated metabolites in fecal extracts. A relationship between high casein diet and urinary excretion of NAG has been described previously (Hallson et al., 1997), which also suggested elevated bacterial proteolysis.

Altogether, our data suggest that the probiotic-induced increased proteolytic activities may reflect the basal metabolism of these Lactobacillus strains, in particular L. paracasei for which the proteolytic activities on casein medium are known to be elevated (Sasaki et al., 1995; Ikram and Mukhtar, 2006). Moreover, increased urinary NAG levels have been reported as a biomarker of increased tubular activity and tubular cell toxicity. Changes in bacterial fermentation of carbohydrates can lead to different ion absorption from the gut (Scholz-Ahrens et al., 2001), which may contribute to altered kidney metabolism and tubular activity. Therefore, the gut microbial contribution to the modulation of the NAG biomarker is certainly of potential toxicological assessment significance.

**H-PCA modelled multicompartamental matrices related to lipid metabolism**

H-PCA has been explored for the first time as a top-down systems approach to model and integrate metabolic profiles from diverse biological compartments. H-PCA modelling summarized clearly the intercorrelated changes induced by probiotic-treatment in plasma, urine and liver matrices and composition in bile acids (Figure 4). One benefit of the hierarchical approach lies in the much simplified simultaneous visualization of global system biochemical changes in multibiological matrices and improves interpretability. For instance, H-PCA resulted in a separation between the treated groups, which was not observed in separate PCA models. In addition, the H-PCA loadings plot gives the relative importance of the different blocks (biological matrices) in carrying diet-induced discriminant information. Moreover, the relationships between the descriptors in the simplified H-PCA space (H-PCA loadings plot) indicate correlations and anticorrelations between block variables, which may give insight into correlations between the metabolic variations in different biological matrices as exemplified here by metabolic changes in liver, plasma and urine and related to lipid metabolism. The H-PCA model also efficiently summarized the intercorrelated changes related to higher systemic glycolysis in plasma, urine and liver matrices, that is, reduced ketone body formation, anaerobic glycolysis, tricarboxylic cycle perturbation and amino-acid catabolism (Figure 4). Such observations might lead to better description of multigorgan metabolic perturbations (Figure 4B). This multicompartamental top-down approach offers a way forward to study the systemic biochemical profiles and regulation of function in whole organisms by analyzing simultaneously several metabolic pools from different biofluids and tissues. This approach also provides a new strategy for the quantitative and qualitative evaluation of different probiotic (or indeed any functional food or nutriceutical) interventions in relation to host biochemistry.

**Conclusion**

Significant associations between host metabolic phenotypes and a nutritionally modified gut-microbiota strongly supports the idea that changes across a whole range of metabolic pathways are the product of extended genome perturbations that can be oriented using probiotic supplementation, and which may play a role in host metabolic health. Bipartite network analysis highlights the metabolic flexibility of the gut microbiota, whereby bacterial strains communicate with each other to metabolize differently bile acids in a gut microbial ecosystem modulated with probiotics. In this case, probiotic consumption exerted a modification over the microbiome resulting in different hepatic influx and efflux of fatty acids in the liver, as observed with increased enterohepatic recycling of bile acids and dietary fats, lowered plasma lipoprotein levels and stimulated glycolysis. Probiotics also induced a different microbial proteolactic activity as well as modulation of bacterial metabolism of amino acids, methyamines and SCFAs. We showed the novel application of H-PCA as a means to study perturbation of metabolic profiles triggered by symbiotic microbiota at a ‘global system’ level by analyzing several metabolite pools simultaneously from different biofluids and tissues. These integrated system investigations demonstrate the potential of metabolic profiling as a top-down systems biology driver for investigating the mechanistic basis of probiotic action and the therapeutic surveillance of the gut microbial activity related to dietary supplementation of probiotics and their health consequences.
Materials and methods

Animal handling procedure

All animal studies were carried out under appropriate national guidelines at the Nestlé Research Center (Lausanne, Switzerland). The HBF is constituted of a total of seven bacterial strains, isolated from stool of a 20-day-old female baby that was naturally delivered and breast-fed, namely *E. coli*, *B. breve* and *B. longum*, *S. epidermidis* and *S. aureus*, *C. perfringens* and *Bacteroides distasonis*. Bacterial cell mixtures contain approximately 10^6 cells/ml for each strain and were kept in frozen aliquots until use. *Lactobacillus paracasei* NCC2461 and *L. rhamnosus* NCC4007 probiotics were obtained from the Nestlé Culture Collection (Lausanne, Switzerland).

A total of 28 female germ-free mice (C3H strain), aged 6 weeks, received a single dose of HBF bacteria mixture and will be called HBF mice in the current manuscript. The experimental design is detailed in Supplementary Figure 2. The animals were fed with a standard pathogen-free rodent diet constituted of 50% cornstarch, 20% casein, 10% sucrose, 7% soybean oil, 5% cellulose, 0.25% choline bitartrate, 0.3% cystine and vitamin and mineral mixtures (Reeves et al., 1993) for 2 weeks. A control group of HBF mice (n=10) received a saline drink ad libitum containing Man, Rogosa and Sharpe (MRS) culture medium and was fed with a basal mix diet containing in composition 2.5% of a glucose-lactose mixture (1.25% each) for 2 additional weeks. Two groups of HBF mice were given a daily probiotic supplement, either *L. paracasei* (group A, n=9) or *L. rhamnosus* (group D, n=9), containing around 10^9 probiotic bacteria in MRS per day mixed with 10% sucrose, 7% soybean oil, 5% cellulose, 0.25% choline bitartrate, 0.3% cystine and vitamin and mineral mixtures (Reeves et al., 1993) for UPLC-MS analysis. Cecal content was kept in frozen aliquots until use.

Microbial profiling of fecal contents

Immediately after collection, fecal pellets were homogenized in 0.5 ml Ringer solution (Oxoid, UK) supplemented with 0.05% (w/v) L-Cystein (HCl). The enumeration of specific microorganisms was performed after plating and incubation of different dilutions of the bacterial solution on selective and semiselective media, for example, *Bifidobacteria* on Eogum Tomato medium, *Lactobacillus* on MRS + antibiotic (phosphomycin, sulmethoxazole, trimethoprim) medium, *C. perfringens* on NN-agar medium, *Enterobacteriaceae* on Drigalski medium and *Bacteroides* on Shaedler Neo Vanco medium. *Enterobacteriaceae* cultures were incubated at 37°C under aerobic conditions for 24 h, and other cultures were incubated under anaerobic conditions over a 48 h period.

Gas-chromatography on cecal content

An aliquot of cecal content was extracted with 4 ml buffer (0.1% (w/v) HgCl_2 and 1% (v/v) H_3PO_4 supplemented with 0.045 mg/ml 2,2-dimethylbutyric acid (as internal standard) per gram fresh weight. The resulting slurry was centrifuged for 30 min at 5000 g at 4°C. Fecal SCFAs were analyzed using a gas-chromatograph (HP 6890) equipped with a flame ionization detector and a DB-FFAP column (J&W Scientific, MSF Friedli & Co. Switzerland) of 30 cm 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. Each sample run was preceded with a cleaning injection of 1.2% formic acid. 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 using external standards (acetate, propionate, iso-butyrate, n-butyrate, iso-valerate, n-valerate) and the concentration was calculated using the internal standard.

1H NMR spectroscopic analysis

A volume of 100 μl of blood plasma was added to 450 μl of saline solution containing 10% D_2O, which was used as a spectrometer field frequency lock, into 5 mm NMR tubes. 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-[1,2,3,3,3-^2H_4] propionate (TSP), which was used as chemical shift reference (δ 0.0), 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 given fecal samples were sonicated for 30 min at 25°C and then centrifuged at 13 000 r.p.m. for 20 min to remove particulates. The supernatants were removed and centrifuged at 13 000 r.p.m. for 10 min. A 580 μl aliquot of the fecal supernatant was then pipetted into a 5 mm NMR tube for spectroscopic analysis. Portions of intact liver samples (~15 mg) were bathed in ice-cold 0.9 % saline D_2O solution and packed into a zirconium oxide 4 mm outer diameter rotor.

1H NMR spectra were acquired for each sample using a Bruker DRX 600 NMR spectrometer (Rheinettenen, Germany) operating at 601.11 MHz for 1H. The instrument was equipped with a Bruker 5 mm TXI triple resonance probe maintained at 298 K for liquid samples and a standard Bruker high-resolution MAS probe under magic-angle-spinning conditions at a spin rate of 5000 Hz for intact tissues (Waters et al., 2000). Tissue samples were regulated at 283 K to minimize biochemical degradation.

One-dimensional (1D) 1H NMR spectra were obtained from each sample using a standard solvent suppression pulse sequence (RD-90°-11.9°-tm-90°-acquire FID) with t_m fixed at 100 ms and t_f at 3 μs (Wang et al., 2005). Additional spin echo Carr-Purcell-Meiboom-Gill (CPMG) spectra were acquired for plasma and liver samples using the pulse sequence (RD-90°-1.180°-1.180°-acquire FID), with a spin–spin relaxation delay, 2nt, of 160 ms for plasma and 200 ms for tissue (Meiboom and Gill, 1958). The 90° pulse length was 9.0–12 μs. A total of 128 transients were collected into 32K data points with a recycle delay (RD) of 2s. The assignment of the 1H NMR spectral peaks to specific metabolites was achieved based on the literature (Nicholson et al., 1995; Fan, 1996), and confirmed by 2D CORelation SpectroscopY (COSY) (Hurd, 1990) and T0tal Correlation SpectroscopY (TOCSY) (Bax and Davis, 1985). 2D NMR spectra were acquired on selected samples. Further assignment of the metabolites was also accomplished with the use of Statistical TOtal Correlation SpectroscopY (STOCSY) on 1D spectra (Cloarec et al., 2005a).

UPLC-MS methods

The Ultra Performance™ liquid chromatography of ileal flushed was performed on a ACQUITY UPLC system (Waters, Milford, MA, USA) equipped with a ToF™ LCT-Premier (Waters MS Technologies, Manchester, UK) for mass detection using the method we described previously (Martin et al., 2007a). The same conditions were applied for analysis of the following bile acid standards: cholic (CA), taurocholic (TCA), glycocholic (GCA), deoxycholic (DCA), taurodeoxycholic (TDCA), glycodeoxycholic (GDCA), chenodeoxycholic (CDCA), taurochenodeoxycholic (TCDCAA), glycochenodeoxycholic (GCDCCA), lithocholic (LCA), tauroliothicolcholic (TTLCA), glycolithocholic (GLCA), ursodeoxycholic (UDCA), tauroursodeoxycholic (TUDCA), glycoursodeoxycholic (GUDCA), hyocholic (HCA), α-muricholic (αMCA), β-muricholic (βMCA), α-muricholic (αMCA), tauro-β-muricholic (TβMCA) acids. We have previously given the molecular structure, the retention time and the mass to charge ratio (and not the molecular weight as published previously) of the observed ions. Unconjugated bile acids formed a formate adduct.

Data analysis

Microbial counts and SCFAs composition in the cecum were analyzed using a two-tailed Mann–Whitney test.
$^1$H NMR spectra were corrected manually for phase and baseline distortion and referenced to the chemical shift of the CH$_3$ resonance of alanine at $\delta$ 1.466 for plasma and liver samples, to the TSP resonance of alanine at $\delta$ 0.0 for urine and fecal samples using XwinNMR 3.5 (Bruker Biospin, Rheinstetten, Germany). The spectra were converted into 22K data points over the range of $\delta$ 0.2–10.0 using an in-house developed MATLAB routine. The regions containing the water resonance ($\delta$ 4.5–5.19), and for urine urea resonance ($\delta$ 4.5–6.2), were removed. Chemical shift intensities were normalized to the sum of all intensities within the specified range before chemometric analysis.

UPLC-MS data were processed using the Micromass MarkerLynx$^{\text{TM}}$ applications manager Version 4.0 (Waters Corp, Milford, USA). The peaks of bile acids were identified by comparing the $m/z$ ratio and retention time to the set of standard bile acids measured under the same conditions. Data were noise-reduced in both of the UPLC and MS domains using MarkerLynx standard routines. Integration of the UPLC-MS bile acid peaks was performed using ApexTrack$^{\text{TM}}$. Each peak integral was expressed as a ratio to the sum of integrals of the 21 measured bile acids.

The multivariate pattern recognition techniques used in this study were based on PCA (Wold et al., 1987), H-PCA (Westerhuis et al., 1998) and the orthogonal-projection to latent structure (O-PLS) (Trygg and Wold, 2003). PCA was carried out using the SIMCA-P+ 11 software (Umetrics, Umeå, Sweden) in order to detect the presence of inherent similarities between metabolic profiles. Both NMR and LC-MS variables were subjected to Pareto scaling, by dividing each variable by the square root of its standard deviation. Data were visualized by means of principal component scores and loadings plots. Each point in the scores plot represents an individual biochemical profile of a sample, whereas on the loadings plot each coordinate represents a single NMR spectral region or LC-MS retention time and mass to charge ratio ($m/z$). Because the scores and loadings plots are complementary, biochemical components responsible for the differences between samples detected in the scores plot can be extracted from the corresponding loadings plot.

O-PLS-DA was also carried out using the method developed by Trygg et al. (Trygg and Wold, 2003) and implemented for NMR spectral data by Cloarec et al. (2005b) to exclusively focus on the effects of probiotic supplementation. All O-PLS-DA models were constructed using one predictive and two orthogonal components using data scaled to unit variance (i.e. by dividing each variable by its standard deviation).

Here, the test for the significance of the Pearson product–moment correlation coefficient was used to calculate the cut-off value of the correlation coefficients at the level of $P<0.05$. To test the validity of the model against over-fitting, the cross-validation parameter correlation coefficient was used to calculate the cut-off value of the model.

The multivariate pattern recognition techniques used in this study were based on PCA (Wold et al., 1987), H-PCA (Westerhuis et al., 1998) and the orthogonal-projection to latent structure (O-PLS) (Trygg and Wold, 2003) and used to detect the presence of inherent similarities between metabolic profiles. Both NMR and LC-MS variables were subjected to Pareto scaling, by dividing each variable by the square root of its standard deviation. Data were visualized by means of principal component scores and loadings plots. Each point in the scores plot represents an individual biochemical profile of a sample, whereas on the loadings plot each coordinate represents a single NMR spectral region or LC-MS retention time and mass to charge ratio ($m/z$). Because the scores and loadings plots are complementary, biochemical components responsible for the differences between samples detected in the scores plot can be extracted from the corresponding loadings plot.

### Supplementary information

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

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