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ORIGINAL ARTICLE

Synbiotic approach restores intestinal homeostasis and prolongs survival in leukaemic mice with cachexia

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Cancer cachexia is a multifactorial syndrome that includes muscle wasting and inflammation. As gut microbes influence host immunity and metabolism, we investigated the role of the gut microbiota in the therapeutic management of cancer and associated cachexia. A community-wide analysis of the caecal microbiome in two mouse models of cancer cachexia (acute leukaemia or subcutaneous transplantation of colon cancer cells) identified common microbial signatures, including decreased *Lactobacillus* spp. and increased *Enterobacteriaceae* and *Parabacteroides goldsteinii* ASF 519. Building on this information, we administered a synbiotic containing inulin-type fructans and live *Lactobacillus reuteri* 100-23 to leukaemic mice. This treatment restored the *Lactobacillus* population and reduced the *Enterobacteriaceae* levels. It also reduced hepatic cancer cell proliferation, muscle wasting and morbidity, and prolonged survival. Administration of the synbiotic was associated with restoration of the expression of antimicrobial proteins controlling intestinal barrier function and gut immunity markers, but did not impact the portal metabolomics imprinting of energy demand. In summary, this study provided evidence that the development of cancer outside the gut can impact intestinal homeostasis and the gut microbial ecosystem and that a synbiotic intervention, by targeting some alterations of the gut microbiota, confers benefits to the host, prolonging survival and reducing cancer proliferation and cachexia.

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Introduction

The trillions of microbes that reside in the gastrointestinal tract of mammals (that is, the gut microbiota) maintain a symbiotic relationship with their host. The gut microbiota has a critical role in biological processes such as maturation of the immune system and host metabolism (Hooper *et al.*, 2012; Brestoff and Artis, 2013). Reciprocally, the host's immune system affords protection against microbes and partially determines the composition of the gut microbiota. This is achieved in part

through the creation of a barrier that involves several factors, including secretion of glycoproteins (mucins), expression of tight junction proteins, release of agents that prevent the translocation of gut microbes (for example, immunoglobulin A) and secretion of antimicrobial proteins such as regenerating islet-derived 3 gamma (Reg3γ), secretory group IIA phospholipase A2 (Pla2g2) and α-defensins (Bevins and Salzman, 2011; Hooper *et al.*, 2012; Mukherjee and Hooper, 2015). The appreciation that the gut microbiota has an essential role in human and animal health has generated tremendous interest in strategies to modulate its composition and metabolic function to benefit the host. Prebiotics are one of these strategies. Prebiotics typically refer to non-digestible compounds that, by being substrates for microorganisms in the gut, modulate the composition and/or activity of the gut microbiota, thus conferring a beneficial physiological

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effect to the host (Gibson and Roberfroid, 1995; Gibson *et al.*, 2004; Bindels *et al.*, 2015). The modulation of the gut microbiome has shown benefits in several experimental disease models, for example, inflammatory bowel diseases, obesity, type 2 diabetes, hepatic steatosis, gastrointestinal cancers, and allergic airway disease (Schwabe and Jobin, 2013; Tilg and Moschen, 2014; Bindels *et al.*, 2015).

The potential role of gut microbes in carcinogenesis and cancer progression in the gastrointestinal tract has been an object of research for decades (Schwabe and Jobin, 2013; Louis *et al.*, 2014). Recently, it has been demonstrated that the microbiome could also impact the occurrence and progression of cancer at extra-intestinal sites (Schwabe and Jobin, 2013). Reciprocally, it has been shown in rodent models that the gut microbiota is altered by cancers that occur outside the gastrointestinal tract (Lin *et al.*, 2012; Bindels *et al.*, 2012a,b). Because the gut microbiota participates in the regulation of host immunity and metabolism, it is of crucial importance from a therapeutic perspective to determine whether it has a role in tumour progression and associated metabolic disorders (often referred to as cancer cachexia) and to further elucidate the mechanisms underlying such interactions.

Cachexia is a serious but often neglected consequence of many chronic diseases. Its prevalence ranges from 5% to 15% of patients in end-stage chronic heart failure up to 50–80% of patients with advanced cancer (Argiles *et al.*, 2014; von Haehling and Anker, 2014). Cancer cachexia is characterized by a loss of fat and muscle masses that is often accompanied by anorexia and inflammation (Argiles *et al.*, 2014). Cancer cachexia remains an unmet medical need, and its treatment will likely require a multimodal therapeutic approach that combines nutritional support with exercises and pharmacological agents targeting the breakdown of skeletal muscle and inflammation (Fearon *et al.*, 2012, 2013). We have previously shown that the administration of specific *Lactobacillus* strains or non-digestible carbohydrates fermented by gut microbes can reduce systemic inflammation and/or cancer progression (Bindels *et al.*, 2012a,b), suggesting that the gut microbiome might be a potential therapeutic target for cancer cachexia. These studies raised several questions: how is the composition of the gut microbiota affected in cancer cachexia? Do the gut microbiota and the intestine have a role in cancer cachexia? What are the mechanisms involved in this crosstalk?

In this study, we used next-generation sequencing, metabolomics and molecular profiling of the host to gain in-depth insight regarding the impact of cancer and cachexia on the composition of the gut microbiota and to identify pathways that are potentially involved in these gut microbiota–host interactions. Using these approaches, we identified bacterial taxa that were associated with alterations of the intestinal homeostasis. The information obtained in the first studies was used to design a dietary approach

combining the oral administration of a bacterium that is known to be decreased in cancer cachexia (*Lactobacillus reuteri*) and a prebiotic (inulin-type fructans, ITF). We then determined the effect of this synbiotic treatment on lifespan and other physiological hallmarks of cancer cachexia.

Materials and methods

Cell culture

Colon carcinoma 26 (C26) cells were maintained in DMEM high-glucose medium supplemented with 10% fetal bovine serum (PAA clone, PAA, Pasching, Austria), 100 µg ml⁻¹ streptomycin and 100 IU ml⁻¹ penicillin (Gibco, Ghent, Belgium) at 37 °C with 5% CO₂. The Pro-B lymphocyte (BaF3/BaF) cell line transfected with Bcr-Abl was maintained as previously described (Bindels *et al.*, 2012a).

Bacterial culture and in vitro testing

L. reuteri was grown at 37 °C in MRS medium (Difco, Franklin Lakes, NJ, USA) until the stationary phase and then washed. For peripheral blood mononuclear cell (PBMC) stimulation, the bacteria were suspended in phosphate-buffered saline containing 20% glycerol and stored at –80 °C until use. For the *in vivo* experiments, bacterial culture was centrifuged, supernatant removed and pelleted bacteria stored at –80 °C. Bacterial numerations were checked after thawing, and the bacteria were suspended at an adequate concentration. PBMCs were isolated from the blood of healthy donors as described previously (Foligne *et al.*, 2007). Briefly, after Ficoll gradient centrifugation (Pharmacia, Stockholm, Sweden), mononuclear cells were collected, washed in RPMI-1640 medium (Life Technologies, Ghent, Belgium), and adjusted to 2 × 10⁶ cells per ml in RPMI supplemented with gentamicin (150 g ml⁻¹), L-glutamine (2 mM), and 10% fetal calf serum (Gibco). PBMCs were stimulated with phosphate-buffered saline or bacteria (bacterium:cell ratio of 10:1) for 24 h at 37 °C with 5% CO₂. The supernatants were collected and stored at –20 °C until cytokine measurements were performed using R&D DuoSet ELISA kits (R&D, Minneapolis, MN, USA).

Animals

Mice from Charles River Laboratories (Chatillon-sur-Chalaronne, France) were housed at two mice per cage with a 12-h light/dark cycle and fed an irradiated normal chow diet (AO4-10, 2.9 kcal g⁻¹, Safe, Augy, France). Six to ten mice were included in each group, as indicated in the legends.

C26 model. Either a saline solution or C26 cells (1 × 10⁶ cells in 0.1 ml saline) were injected subcutaneously (intrascapular) in male CD2F1 mice (8 weeks old).

BaF model. Either a saline solution or BaF cells (1 × 10⁶ cells in 0.1 ml saline) were injected into

the tail vein of anesthetized female Balb/c mice (6 weeks old). The dietary restriction experiment is described in the Supplementary information. For the synbiotic treatment, 1 day after the BaF injection, the mice received *Lactobacillus reuteri* 100-23 (2×10^8 CFU per ml) with 0.2 g per day of ITF (oligofructose, Orafit p95, 1.5 kcal g^{-1} , Beneo-Orafti, Oreye, Belgium) in the drinking water, as previously described (Bindels *et al.*, 2012a, b). The drinking solutions were replaced every day, and food and water intake were recorded. Synbiotic mice consumed on average 5.8×10^8 CFU per day for the main experiment and 6.34×10^8 CFU per day for the survival experiment (Supplementary Figure S1). Energy intake was calculated taking into account the energy content of the diet and energy from ITF.

At the end of the experiment (11 days for the C26 model and 13 days for the BaF model), portal blood and tissue samples were harvested following anaesthesia (ketamine and xylazine, or isoflurane gas, Abbot, Wavre, Belgium). Portal blood was collected in EDTA tubes and centrifuged (13 000 g, 3 min). Tissues were weighed and frozen in liquid nitrogen. All of the samples were stored at -80°C .

For the survival experiment, a separate set of mice was used. Half the mice received the synbiotic treatment, as described above. A morbidity score was performed on day 14 as described in the Supplementary information. Mice were monitored every 3–6 h from day 15 to day 19. All mice died from cancer, and therefore, no tissues and blood were withdrawn.

The experiments were approved by the ethics committee of the Université catholique de Louvain, and the housing conditions were consistent with the specifications of the Belgian Law of 29 May 2013 for the protection of laboratory animals (Agreement LA 1230314).

Tissue mRNA analyses

The isolation of RNA, preparation of complementary cDNA and real-time PCR were performed as previously described (Bindels *et al.*, 2012a). The RNA quality was checked using an Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA). The primer sequences are detailed in Supplementary Table S1. Full description of the quantitative real-time PCR (qPCR) procedures is provided in the Supplementary information.

Gut microbiota analyses

Genomic DNA was extracted from the caecal content using a QIAamp DNA Stool Mini Kit (Qiagen, Hilden, Germany), including a bead-beating step. Absolute quantification of the bacterial taxa was performed by qPCR (primers presented in Supplementary Table S1). Full description of the qPCR procedures is provided in the Supplementary information. Illumina sequencing was performed as previously described (Krumbeck *et al.*, 2015). The

V5-V6 region of the 16S rRNA gene was amplified by PCR with modified primers. The amplicons were purified, quantified and sequenced using an Illumina Miseq to produce 2x300-bp sequencing products at the University of Minnesota Genomics Center. Initial quality-filtering of the reads was conducted with the Illumina Software, yielding an average of 148 370 pass-filter reads per sample. Quality scores were visualized and reads were trimmed to 240 bp. The reads were merged with the merge-Illumina-pairs application (Eren *et al.*, 2013). For samples with >50 000 merged reads, a subset of 50 000 reads was randomly selected using Mothur 1.32.1 (Schloss *et al.*, 2009) to avoid large disparities in the number of sequences. Subsequently, the UPARSE pipeline implemented in USEARCH v7.0.1001 (Edgar, 2013) was used to further process the sequences. Putative chimaeras were identified against the Gold reference database and removed. Clustering was performed with a 98% similarity cutoff to designate operational taxonomic units (OTUs). Non-chimaeric sequences were also subjected to taxonomic classification using the RDP MultiClassifier 1.1 from the Ribosomal Database Project (Cole *et al.*, 2014) for phylum to genus characterization of the faecal microbiome.

The phylotypes were computed as percent proportions based on the total number of sequences in each sample. Alpha- and beta-diversity indexes were calculated using QIIME (Caporaso *et al.*, 2010). The LDA effect size was computed and plotted using LEfSe (Segata *et al.*, 2011). The complete protocol, statistical analysis and accession numbers are provided in the Supplementary materials and methods.

Metabolic profiling by ^1H NMR spectroscopy

In all, 50 μl of portal plasma was mixed with 10 μl of D_2O , vortexed and centrifuged, and the supernatant was transferred into NMR capillary tubes. The complete protocol and statistical analysis are described in the Supplementary materials and methods.

Statistical analyses

The statistical significance of differences between groups was assessed using Student's *t*-test when comparing two groups, or one-way ANOVA followed by Tukey's multiple comparison *post hoc* test when comparing several groups. The data are presented as the mean \pm s.e.m. or as whiskers plots with minima and maxima. $P < 0.05$ was considered as statistically significant. More information is provided in the Supplementary materials and methods.

Results

Cancer development alters the caecal microbiota

Two models of cancer cachexia were used: the BaF model and the C26 model. The BaF model involves

the intravenous injection of Bcr-Abl-expressing lymphocytes, thereby mimicking acute leukaemia (Bindels *et al.*, 2012b). The C26 model consists of a subcutaneous injection of colon 26 (C26) tumour cells (Sciorati *et al.*, 2009; Talbert *et al.*, 2014). Both models are characterized by a loss of fat mass and muscle atrophy, as published previously for the BaF model (Bindels *et al.*, 2012a) and as shown in Supplementary Figure S2 for the C26 model. The caecal microbiota analysis by Illumina sequencing of 16S rRNA tags revealed profound shifts in the composition of the microbiota in both models, as evidenced by principal coordinates analysis of the Morisita-Horn beta-diversity at the OTU level (Figures 1a and b). The three first principal coordinates explained 74% and 95% of the variation for the BaF and C26 models, respectively. Alpha-diversity indexes of richness (observed species), or richness and evenness (Shannon), were decreased in BaF and C26 mice compared with their healthy counterparts (Figures 1c and d). In the BaF and C26 models, 25 and 54 taxa were affected, respectively. The changes common to both models encompassed a decrease in the *Clostridiales* order, *Clostridia* class, *Lactobacillaceae* family and *Lactobacillus* genus, and an increase in the *Enterobacteriaceae* family, *Enterobacteriales* order, *Parabacteroides* genus and *Bacteroidetes* phylum (Figures 1e and f). In the C26 model, using an OTU-based analysis, we identified 70 significantly affected phylotypes. Among others, OTU 17 and OTU 97 (both identified as *Escherichia*) were increased by 2698-fold and 4676-fold, respectively, while the relative abundance of OTU 2 (identified as *Parabacteroides goldsteinii*/ASF 519) increased from 1% to 30% of the total 16S rDNA sequences. OTU 54 (identified as *Lactobacillus johnsonii/gasseri*) showed a four-fold decrease (Supplementary Table S2). qPCR analysis of *Enterobacteriaceae*, *P. goldsteinii*/ASF 519 and *Lactobacillus* spp. shows trends similar to the pyrosequencing results (Supplementary Figure S3).

Cachectic mice exhibited reduced food intake (Supplementary Figure S4). To explore whether the modification of the specific taxa was due to changes in food intake, healthy mice were dietary restricted to mimic the decreased food intake of the BaF mice, and the same taxa were analysed by qPCR (Supplementary Figure S4). This experiment allowed us to conclude that the changes in *Lactobacillus* and *Enterobacteriaceae* that occur in cancer and the associated cachexia were not due to decreased food intake.

This set of experiment led us to the conclusion that, in two mouse models of cancer and cachexia, the gut microbiota is impacted by the development of cancer outside the gastrointestinal tract.

A synbiotic approach reduces the progression of cancer and associated cachexia

To further investigate the therapeutic potential of the gut microbiota, we applied a synbiotic approach by

combining ITF, a well-known prebiotic, and a *Lactobacillus* species. The rationale for the use of dietary lactobacilli was that these bacteria were consistently reduced during cachexia, especially in the BaF model. In addition, we demonstrated in a previous study that the administration of a mixture of *L. gasseri* 311476 and *L. reuteri* 100-23 reduced systemic inflammation and muscle atrophy in the BaF model (Bindels *et al.*, 2012a). *In vitro* co-incubation of each bacterial strain with human PBMCs suggested that *L. gasseri* evoked pro-Th1 responses, whereas *L. reuteri* appeared to possess more anti-inflammatory properties (Figure 2). Therefore, *L. reuteri* 100-23 was selected for the synbiotic approach and combined with ITF.

BaF mice were fed ITF and *L. reuteri* (LrI) throughout the course of the study. Thirteen days after injection, the LrI-treated mice exhibited decreased hepatic accumulation of BaF cells, as evidenced by a reduced liver weight and a lower expression of Bcr-Abl, a chimaeric protein that is specifically expressed in BaF cells (Figures 3a and b). Of note, we previously showed that these two parameters are key markers of BaF cell accumulation in the liver (Bindels *et al.*, 2012b). Bcr-Abl expression in the spleen was not impacted by the synbiotic treatment (not detected in control mice, BaF: 1.00 ± 0.14 ; BaF-LrI: 0.90 ± 0.11 , P -value = 0.59, $n=8$). In addition, LrI resulted in the improvement of some cachectic features such as muscle mass loss and the expression of atrophy markers in tibialis and gastrocnemius muscles (Figures 3c and e). In this experiment, synbiotic treatment did not significantly impact the daily energy intake (Figure 3f).

A separate set of mice was used to investigate whether synbiotic treatment can affect morbidity and mortality. LrI administration lowered the morbidity score by half and prolonged the survival of mice by 2 days (Figures 3g and h). In this experiment, no change in energy intake was observed following the administration of LrI, except on days 1 and 12 (Supplementary Figure S5).

The synbiotic approach changes the gut microbiota composition

Synbiotic administration led to higher weights of caecal content and tissue (Supplementary Figure S5). Illumina sequencing confirmed profound shifts in the structure of the microbiota associated with cancer development, and it revealed that the synbiotic approach counteracted some of those changes (Figures 4 and 5, Supplementary Figure S5, Supplementary Tables S3 and S4). At the genus level, BaF injection was associated with the enrichment of *Parabacteroides*, *Clostridium* XI, *Anaerotruncus* and *Escherichia*, and the reduction of *Barnesiella*, *Lactobacillus*, *Blautia*, *Clostridium* XIVb and *Parasutterella*, among others; several of these changes were observed in previous experiments (Figures 4 and 5, Supplementary Figure S6 and Supplementary Table S3). LrI reversed the increased

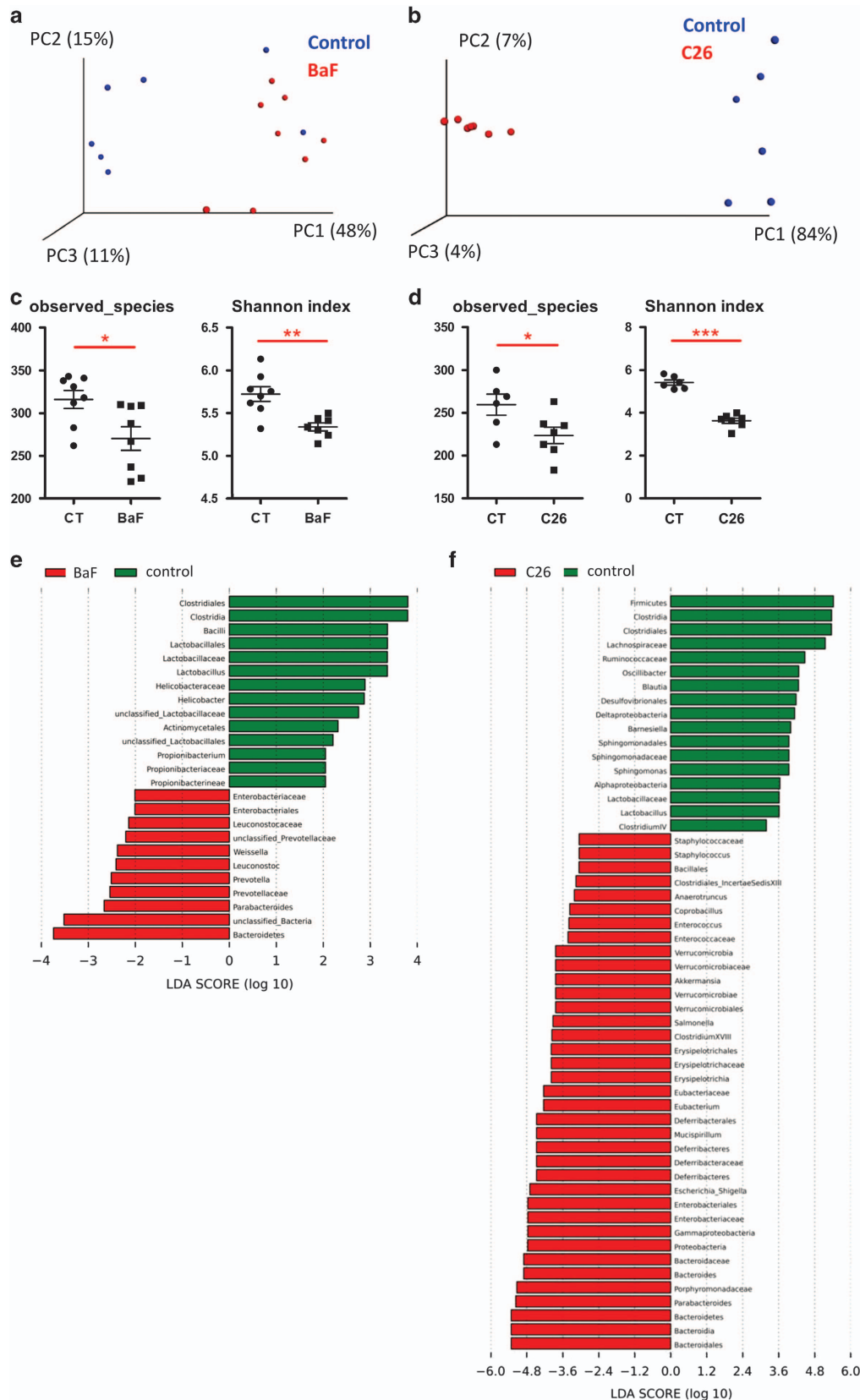


Figure 1 Shifts in the composition of the gut microbiota in the BaF and C26 models. **(a, b)** Principal coordinate analysis of the Morisita-Horn beta-diversity index computed based on the OTU table. **(c, d)** Alpha-diversity indexes in cancer-bearing mice vs controls (CT). The observed species represent an index of richness, and the Shannon index takes into account the richness and evenness. **(e, f)** LDA scores in red for the taxa enriched in cancer-bearing mice and in green for the taxa enriched in control mice. $N=6-8$, $*P<0.05$, $**P<0.01$, $***P<0.001$.

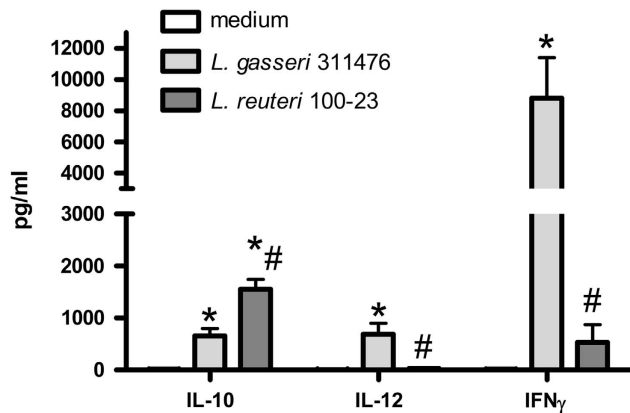


Figure 2 *L. gasseri* evokes pro-Th1 responses, whereas *L. reuteri* appears to possess more anti-inflammatory properties. Cytokine levels in the medium after co-culture of human PBMC and *Lactobacillus* species (two independent experiments involving a total of nine PBMC donors). * $P < 0.01$ vs medium, # $P < 0.01$ vs *L. gasseri* 311476.

abundances of *Escherichia* and *Anaerotruncus*, whereas it increased the relative abundances of the *Coriobacteriaceae* family, *Clostridium* cluster XVIII, and some genera such as *Barnesiella*, *Lactobacillus*, *Bifidobacterium* and *Parasutterella* (Figures 4 and 5, Supplementary Figure S6). The OTU-based analyses confirmed these results and indicated a five-fold increase in the relative abundance of the segmented filamentous bacteria (SFB, OTU 185) (Figure 5, Supplementary Figure S7 and Supplementary Table S4). Quantification of *Enterobacteriaceae*, *P. goldsteinii*/ASF 519 and *Lactobacillus* spp. by qPCR confirmed the pyrosequencing results (Figures 5d–g).

Of note, five of the eight bacterial taxa previously found to be commonly affected in both BaF and C26 models were similarly influenced by cancer development in this new set of experiments (namely, the *Lactobacillaceae* family, *Lactobacillus* genus, *Enterobacteriaceae* family, *Enterobacteriales* order

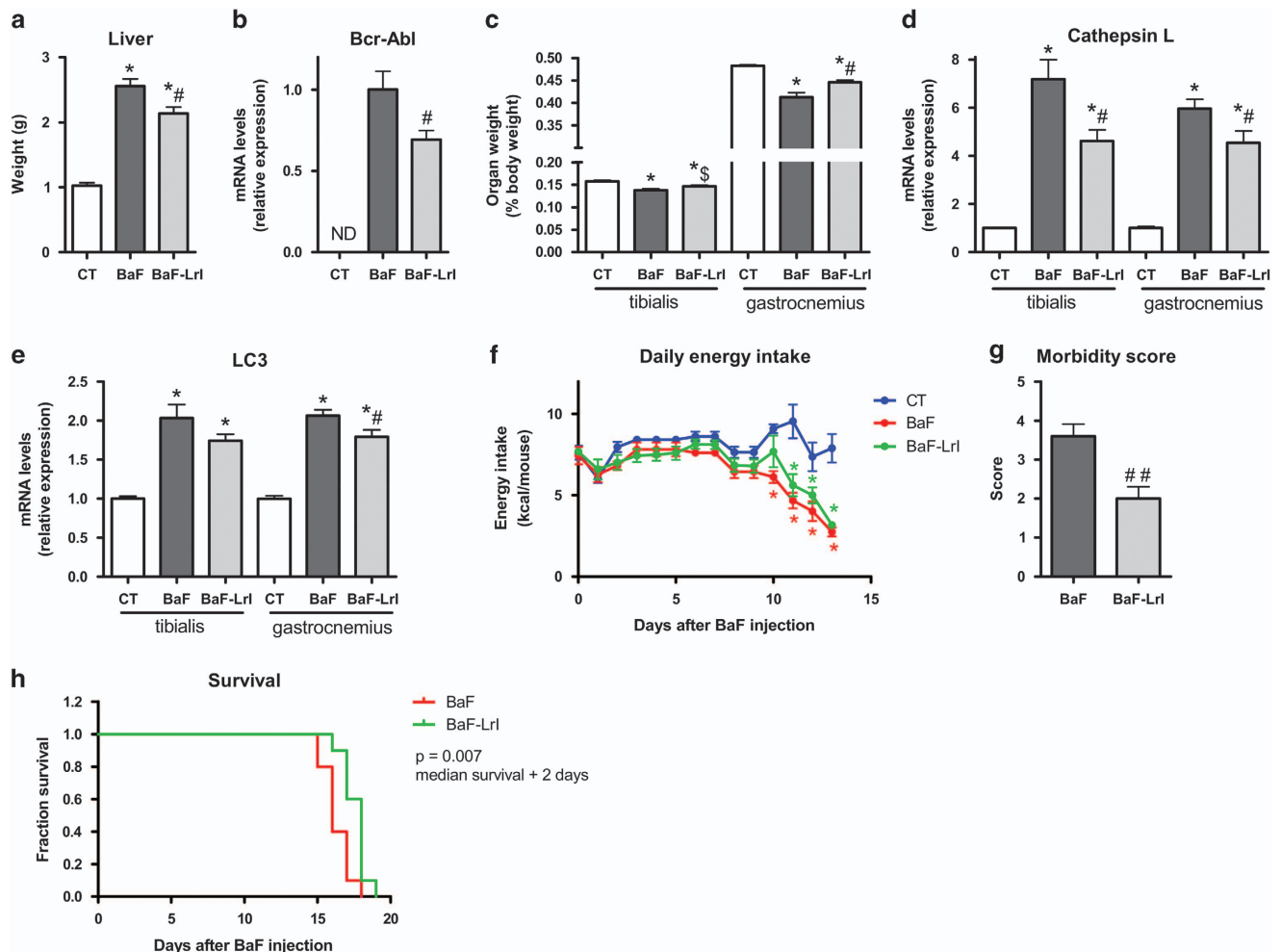


Figure 3 The synbiotic approach reduces hepatic cancer cell accumulation and cachectic features in leukaemic mice. (a) Liver weight. (b) Bcr-Abl mRNA expression in the liver. ND, not detected. (c) Muscle weights. (d, e) mRNA expression of atrophy markers in the tibialis and gastrocnemius muscle. (f) Daily energy intake. (g) Morbidity score performed at day 14. (h) Kaplan–Meier curve showing the survival fractions analysed by the log-rank test ($P = 0.007$). $N = 8$ for (a–f) and $n = 10$ for (g) and (h). * $P < 0.05$ vs control, # $P < 0.05$ vs BaF, ## $P < 0.01$ vs BaF, § $P = 0.06$ vs BaF.

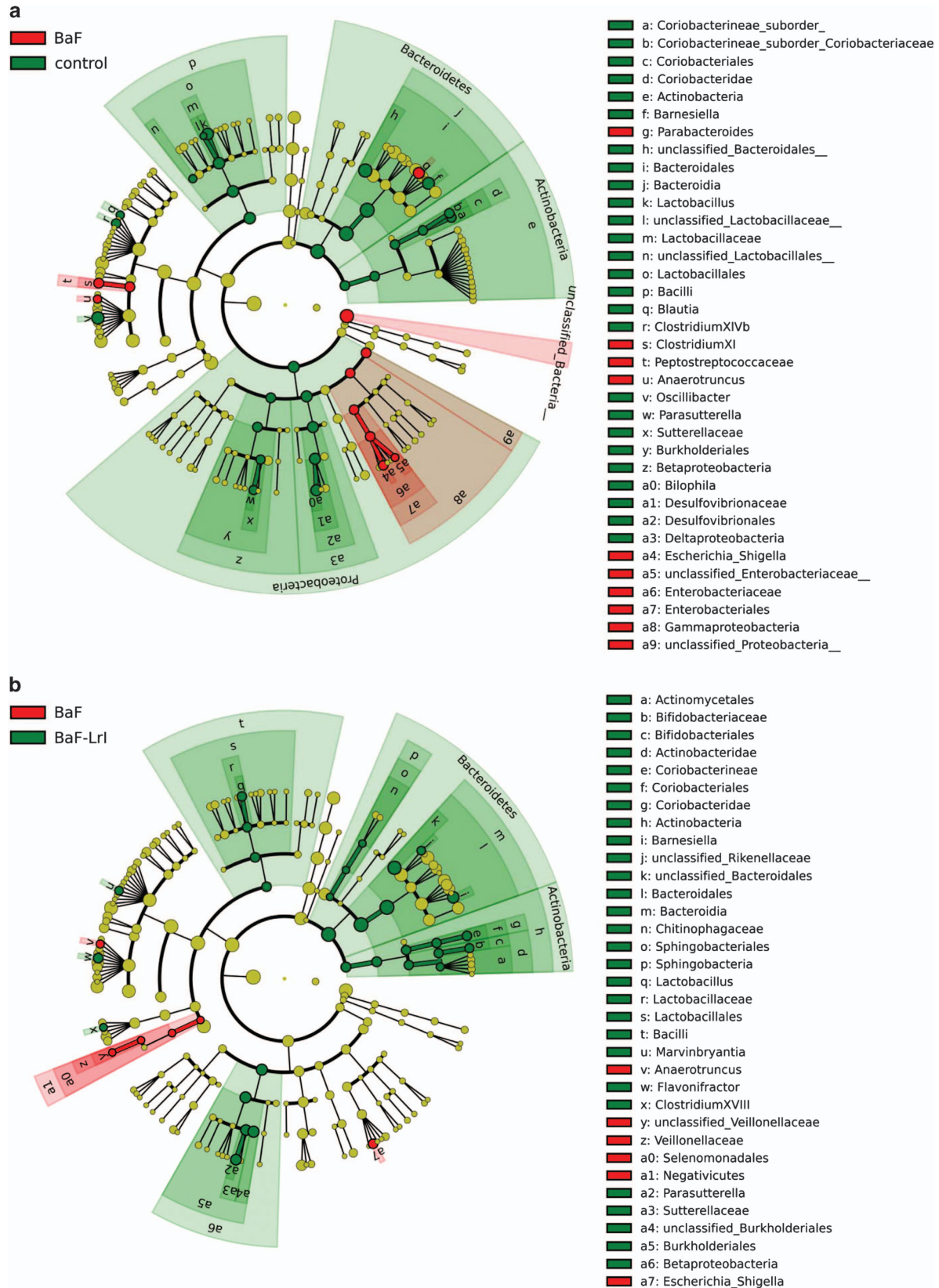


Figure 4 The synbiotic approach changes the gut microbiota composition. **(a)** LEfSe cladogram in red for the taxa enriched in BaF mice and in green for the taxa enriched in control mice. **(b)** LEfSe cladogram in red for the taxa enriched in BaF mice and in green for the taxa enriched in BaF-LrI mice. $N=8$.

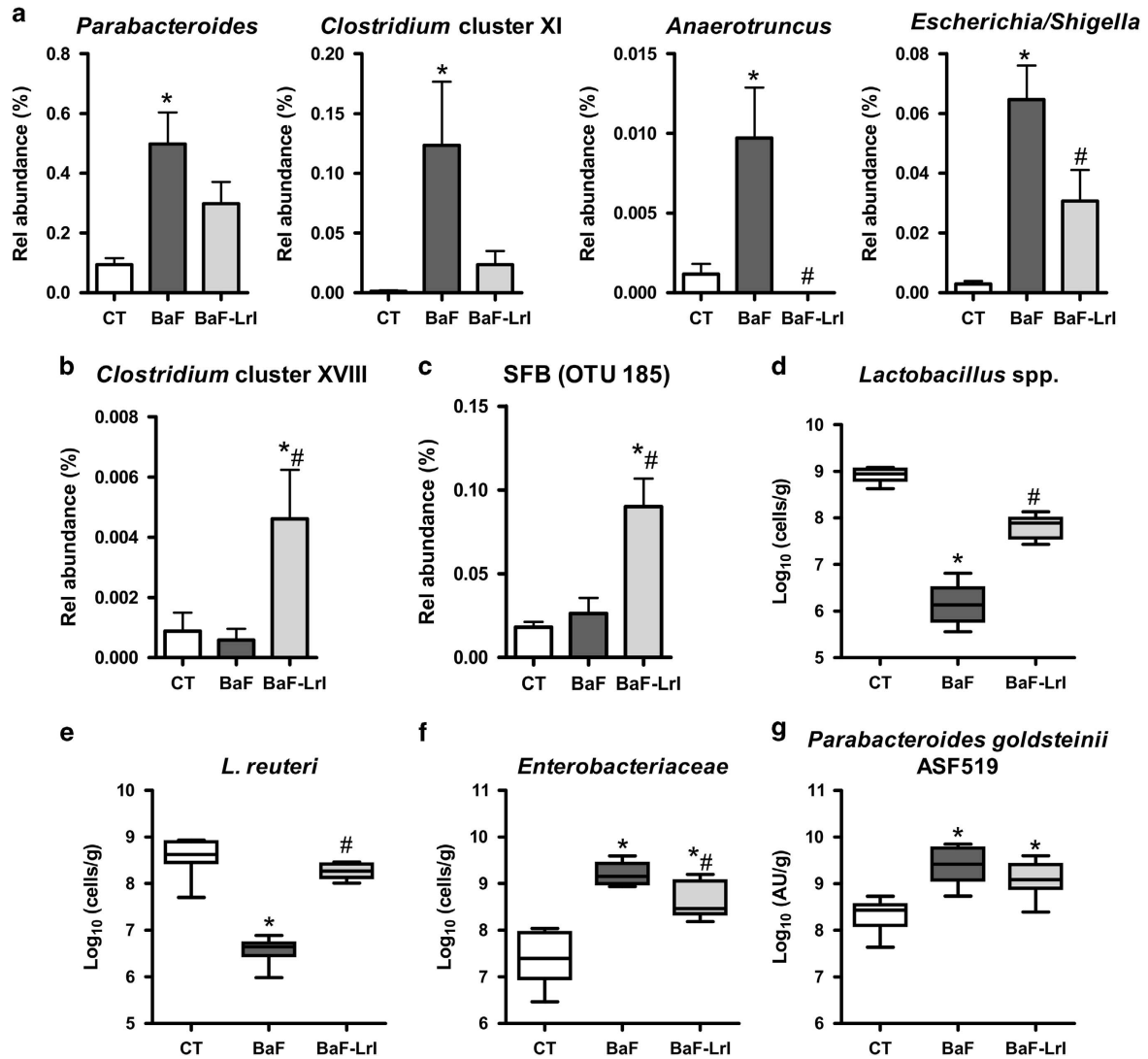


Figure 5 The synbiotic approach changes the abundance of several bacterial taxa. (a) Species enriched in BaF mice according to the LEfSe analysis. (b) *Clostridium* cluster XVIII. (c) SFB/OTU 185. (d–g) Bacteria levels in the caecal content of BaF mice fed or not the synbiotic, as determined by qPCR. AU, arbitrary units. $N=8$, * $P<0.05$ vs control, # $P<0.05$ vs BaF.

and *Parabacteroides* genus) (Figure 4 and Supplementary Figure S6). This microbial signature (increase in the levels of *Enterobacteriaceae* and *P. goldsteinii*/ASF 519 and decrease in *Lactobacillus* levels) was also observed in two additional BaF experiments performed 18 months apart, demonstrating the reproducibility of our findings (Supplementary Figure S8).

Cancer progression changes the portal metabolome

To better understand how changing the composition and activity of the gut microbiota could reduce cancer cell proliferation and the underlying cachexia, we performed a metabolomics analysis of the portal blood of mice. Principal coordinate analysis of the metabolic profiles revealed clear clustering of the control mice due to the enrichment of glucose, fumarate and lipoproteins in the control group and of lactate,

creatine and succinate in the BaF and BaF-LrI groups (Figure 6). A pairwise comparison of the NMR profiles of BaF and BaF-LrI mice did not highlight discriminant metabolites. O-PLS regressions on significantly affected OTUs and PCR transcripts revealed that plasma lipoproteins were negatively associated with the presence of some microorganisms, such as an unclassified *Porphyromonadaceae* (OTU 88) and the *Enterobacteriaceae* family (Supplementary Figure S9).

Synbiotic-mediated improvements in intestinal immune and barrier biomarkers correlate with microbial changes

To identify putative mechanistic links between microbial dysbiosis and cancer and cachexia, we characterized the gut microbiota–host crosstalk at the intestinal level. Antimicrobial peptides greatly affect the composition of the gut microbiota

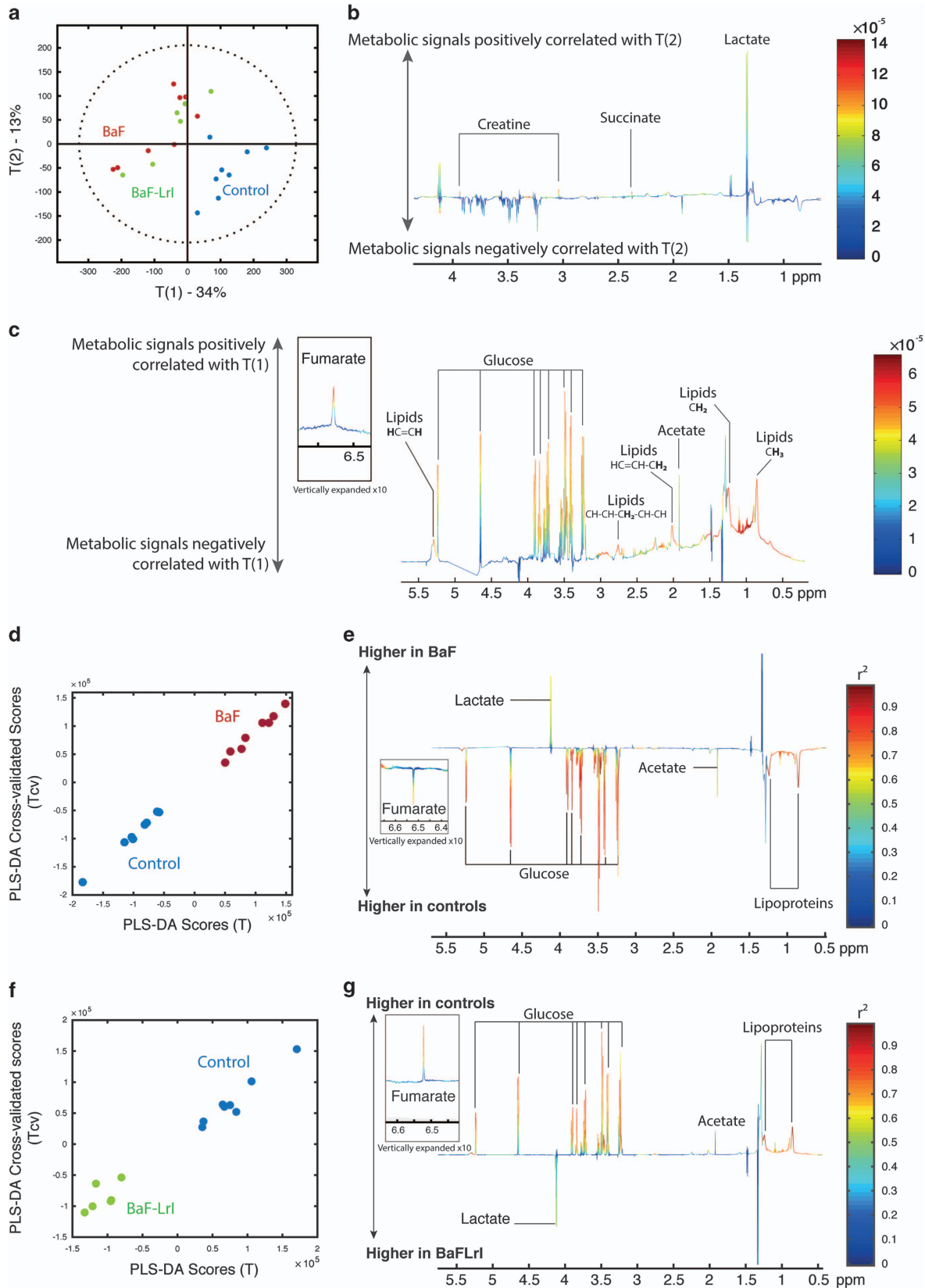


Figure 6 Cancer progression changes the portal metabolome. (a) Principal coordinate analysis plot of the metabolomics profiles derived from portal plasma of control (blue), BaF (red) and BaF-LrI (green) mice, and associated loadings of PC2 (b) and PC1 (c). Pairwise O-PLS-DA comparison showing the calculated scores (x axis) against the cross-validated scores (y axis) and their associated loading coefficients. The weights of the variables are color-coded according to the square of the O-PLS-DA correlation coefficients. (d, e) Controls vs BaF and (f, g) controls vs BaF-LrI. $N=6-8$.

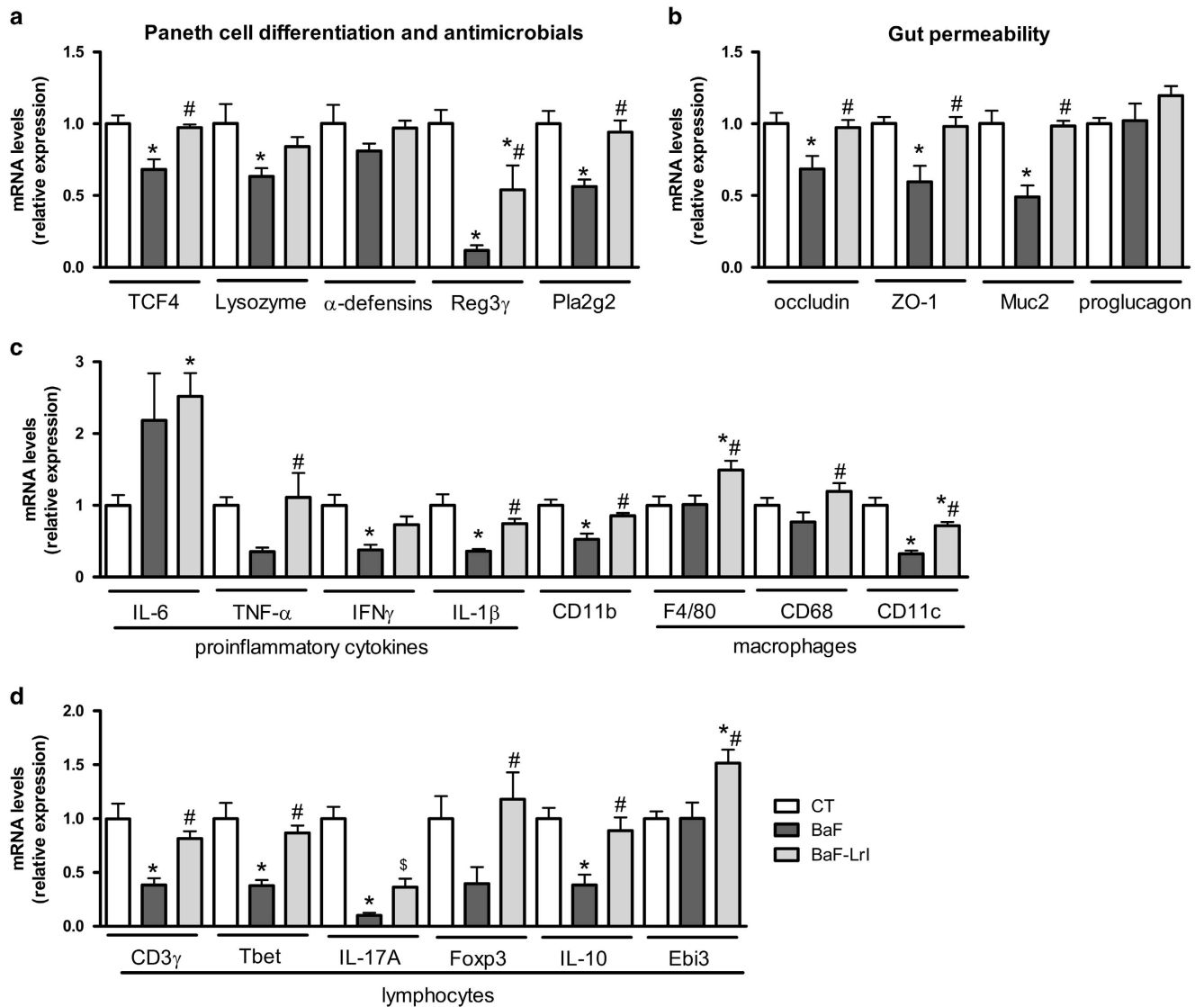


Figure 7 The synbiotic approach improves intestinal immune and barrier biomarkers in leukaemic mice. **(a)** mRNA expression of markers involved in Paneth cell differentiation and antimicrobial proteins. **(b)** mRNA expression of markers involved in gut permeability. **(c)** mRNA expression of immune markers related to inflammation, innate immunity (CD11b) and macrophages. **(d)** mRNA expression of immune markers related to lymphocytes (CD3 γ) Th1 (Tbet), Th17 (IL-17A) and Treg (Foxp3, IL-10, Ebi3) cells. $N=7-8$, * $P<0.05$ vs control, # $P<0.05$ vs BaF.

(Bevins and Salzman, 2011). Here, the expression of antimicrobial proteins such as Lysozyme 1, Reg3 γ and Pla2g2 was decreased in BaF mice, as well as the expression of TCF4 (T cell-specific transcription factor 4), which drives Paneth cell differentiation (Figure 7a) (Bevins and Salzman, 2011). Whereas antimicrobials constitute a chemical barrier against pathogens and commensals, tight junction proteins, such as zonula occludens 1 (ZO-1) and occludin, and mucus secretion participate to construct a physical barrier that limits host exposure to gut microbes. BaF cell injection decreased the expression of occludin, zonula occludens 1 and Muc2 (Figure 7b). The expression levels of immune markers of inflammation (TNF- α , IFN- γ and IL-1 β), the innate immune system (CD11b), proinflammatory macrophages

(CD11c), T lymphocytes (CD3 γ), Th1 lymphocytes (Tbet) and Th17 lymphocytes (IL-17A) were also decreased in the presence of cancer, whereas IL-6 and the macrophage pan-markers F4/80 and CD68 remained unaffected (Figures 7c and d). Interestingly, the administration of LrI counteracted most of the BaF-induced alterations detected in the intestine. Although ITF have been shown to improve gut barrier function through an increase in the production of glucagon-like peptide 2 (Cani *et al.*, 2009), LrI administration did not increase the mRNA expression of proglucagon, the precursor of glucagon-like peptide 2 in this study (Figure 7b).

Regulatory T (Treg) cells, a subset of lymphocytes that restrain the immune response, contribute to intestinal and systemic immune homeostasis and

can be induced by commensal microbiota (Rubtsov *et al.*, 2008; Arpaia *et al.*, 2013; Atarashi *et al.*, 2013). Treg development is controlled by the transcription factor Foxp3, while the function of Tregs is mediated by IL-35 and IL-10 (Collison *et al.*, 2007; Gavin *et al.*, 2007). Intestinal Foxp3 and IL-10 expression was reduced in BaF mice and restored following LrI treatment, whereas the expression of Ebi3, a subunit of the heterodimeric cytokine IL-35, was not impacted by cancer but was induced in response to LrI (Figure 7d). The immunomodulatory effect of LrI was not limited to the gastrointestinal tract. Indeed, LrI administration increased Foxp3 mRNA levels in the spleen (BaF: 1.0 ± 0.1 ; BaF-LrI: 2.0 ± 0.4 , $P=0.047$), and this increase correlated with the relative abundance of *Clostridium* Cluster XVIII (Pearson $r=0.71$, $P=0.002$), a group of microbes that is able to induce Tregs (Atarashi *et al.*, 2013).

Finally, a multiple correlation analysis between intestinal biomarkers and phylotypes that were significantly affected highlighted *Escherichia* sp. and *P. goldsteinii*/ASF 519 as two bacteria that correlated inversely with intestinal alterations. In addition, several OTUs, encompassing mainly *Barnesiella*, *Lactobacillus*, *Enterorhabdus* and unclassified *Porphyromonadaceae* species, correlated with Reg3 γ , CD11b, CD11c and CD3 γ expression (Figure 8, Supplementary Table S5).

Discussion

In this study, we demonstrated that the gut microbial balance was altered by the presence of tumours located outside the gastrointestinal tract, consistently with our previous study where we provided the first evidence of a modified gut microbiome in a mouse model of leukaemia and cachexia (BaF) (Bindels *et al.*, 2012a). Caecal *Lactobacillus* spp. levels were decreased in leukaemic mice with cachexia, and a PCR-DGGE (denaturing gradient gel electrophoresis) analysis suggested that bacteria other than lactobacilli were affected by the disease. The same year, Lin *et al.* (2012) reported that subcutaneous implantation of colon cancer cells in rats was associated with a one-log increase in *Enterobacteriaceae* and *Clostridium* cluster I and XI in the faeces. In both studies, microbial dysbiosis was assessed using molecular approaches with limitation in dynamic range. Here we used a community-wide molecular approach to deeply characterize the microbial shifts occurring in two mouse models of cancer cachexia. Our data revealed a microbial signature common to both models that included the *Enterobacteriaceae* family/*Escherichia* genus, *Lactobacillus* genus and *Parabacteroides goldsteinii*/ASF 519 species. Some of these changes have also been observed in the previous studies (Lin *et al.*, 2012; Bindels *et al.*, 2012a), and have been reported as a sign of reduced colonization resistance (Lawley and Walker, 2013).

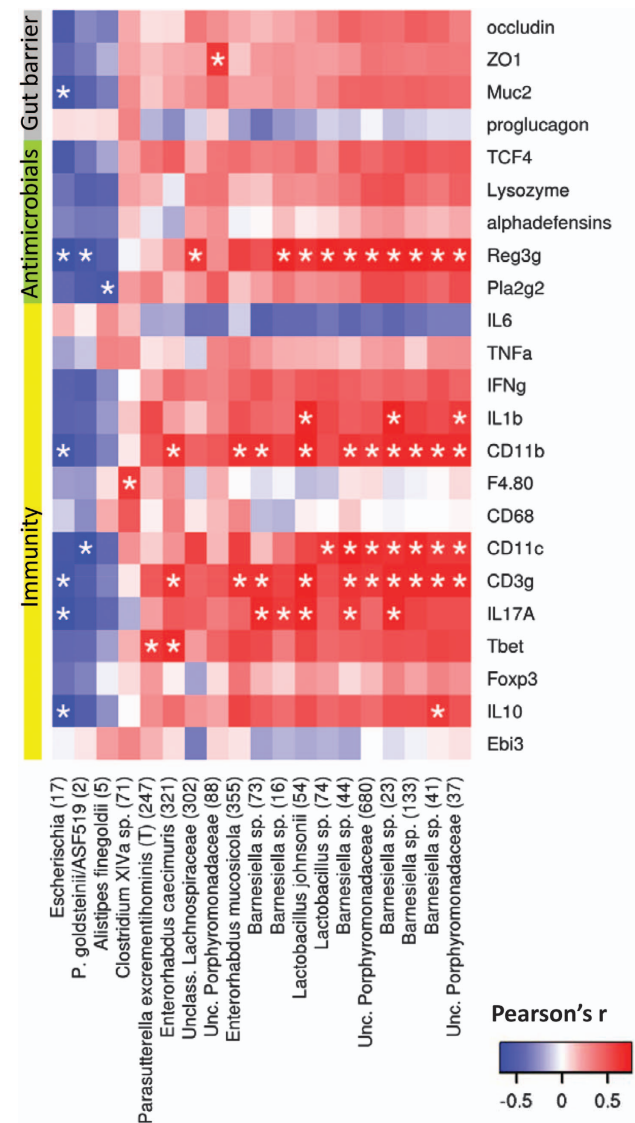


Figure 8 Synbiotic-mediated improvements in intestinal immune and barrier biomarkers correlate with microbial changes. Heat map representation of the Pearson's r correlation coefficient between intestinal biomarkers and significantly affected phylotypes. The closest known taxon is indicated with the OTU number within brackets. *Adjusted $P < 0.05$.

One potential mechanism for the dysbiosis observed during cachexia in mice might be the associated reduction in food intake observed at the late stage of the disease. Food intake has been reported to influence the composition of the gut microbiota (Crawford *et al.*, 2009), and it may be considered that this reduced food intake constitutes a confounding factor in this mouse model. However, one must consider that anorexia is a fully fledged hallmark of cachexia, and thus, it should be integrated into experimental models. Our experiments revealed that the major shifts (*Enterobacteriaceae* and lactobacilli) occurred independently of food intake, suggesting that the presence of cancer cells impacts the gut microbial ecology

independently of food intake. Nonetheless, even if anorexia contributes to dysbiosis, it would not impair the therapeutic implication of our findings. Synbiotic treatment modified to a very limited extent daily energy intake of cachectic mice in one out of two experiments. We can consider that the impact is minor compared with the leukaemia-induced reduced energy intake and likely not relevant enough to be taken into consideration for the interpretation of the data.

Our study results clearly demonstrated gut microbiota alterations in two different models of cachexia. These findings raise the question of whether this dysbiosis contributes to the observed pathologies. We have previously demonstrated the therapeutic interest of the restoration of lactobacilli (Bindels *et al.*, 2012a). To our knowledge, the role of microorganisms, such as *Escherichia* and *P. goldsteinii*/ASF 519, in cancer cachexia has not been investigated yet. *P. goldsteinii*/ASF 519 is a mouse commensal species that is also found at low levels in humans (Dewhirst *et al.*, 1999; Song *et al.*, 2005). The ELDERMET study (Claesson *et al.*, 2012) indicated that individuals in long-term care developed dominant levels of *Parabacteroides* and *Alistipes* that were associated with changes in frailty, inflammation and altered abundances of short-chain fatty acid producers. Similarly to *P. goldsteinii*, the genus *Escherichia* encompasses rodent and human commensal strains and is present at low levels in healthy subjects. Some strains are beneficial to the host, whereas others have been associated with inflammatory bowel disease and colorectal cancer (Manichanh *et al.*, 2012; Louis *et al.*, 2014; Sha *et al.*, 2014). In our study, the synbiotic approach reduced the levels of *Enterobacteriaceae*. This phenomenon could be mediated by short-chain fatty acids (acetate, propionate and butyrate), the production of which is fostered by ITF (Bindels *et al.*, 2012b; Everard *et al.*, 2014), and which, in association with a decreased caecal pH, create a non-permissive environment for colitogenic *Enterobacteriaceae* (Veiga *et al.*, 2010).

Additional research is needed to establish whether the gut microbiota has a role in cancer cachexia. Yet, based on our findings, the following hypothesis can be formulated. Systemic inflammation due to cancer development will alter the gut permeability, antimicrobial defence and Paneth cell function, leading to dysbiosis. Thereafter, the altered intestinal homeostasis, microbial dysbiosis and cancer cachexia would interact in a vicious cycle, where cancer-induced alterations of intestinal homeostasis foster the growth of certain bacteria that further increase systemic inflammation and cachexia. This hypothesis is supported by several elements, independently of the data presented herein. First, systemic inflammation, which has a key role in cancer cachexia (Fearon *et al.*, 2012, 2013; Argiles *et al.*, 2014; von Haehling and Anker, 2014), is present in BaF and C26 mice (Fearon *et al.*, 2012; Bindels *et al.*, 2012a, b) and increases epithelial

permeability (Ivanov *et al.*, 2010). Moreover, inflammation boosts the growth of *Enterobacteriaceae* and *E. coli* and creates an opportunity for *E. coli* to adhere to the colonic mucosa by decreasing the production of mucins and antimicrobials (Lupp *et al.*, 2007; Winter *et al.*, 2013; Louis *et al.*, 2014). Finally, a similar relationship among Paneth cells, the loss of bacterial diversity, *E. coli* blooming and immune alterations has recently been reported in other mouse models (Inaba *et al.*, 2010; Arthur *et al.*, 2012; Eriguchi *et al.*, 2012).

In addition to establishing the presence of microbial shifts in cancer cachexia, the results of the present study clearly provided evidence for considering the gut microbiota as a therapeutic target in cancer and cachexia. Indeed, nutritional modulation of the microbiome by combined pre and probiotic approaches resulted in the reduction of cancer cell proliferation and an improvement of the cachectic features, thus leading to prolonged survival. As *L. reuteri* is not able to metabolize ITF, this combination would be called a complementary synbiotic approach (Kolida and Gibson, 2011). Our data obtained *in vitro* confirmed that *L. reuteri* 100-23 is rather anti-inflammatory. Therefore, one could speculate that such immune modulation could foster cancer proliferation (Mantovani *et al.*, 2008). However, this was not observed here.

To unravel the mechanisms by which the synbiotic approach provides its benefits, we performed 16S sequencing of the gut microbiota, metabolomic analysis of the portal blood and molecular characterization at the intestinal level, which allowed us to draw some hypotheses regarding the mechanism(s) underlying the benefits of the synbiotic treatment.

First, the synbiotic approach could mediate its benefits through the restoration of intestinal homeostasis. The implication of elevated gut permeability in cancer cachexia has been recently proposed (Puppa *et al.*, 2011; Bindels and Delzenne, 2013). Our data clearly showed that cancer cells implanted at a distance from the gastrointestinal tract can affect the gut barrier and immune function. The changes in gut barrier function may contribute to the inflammatory status and thereby to the cachectic phenotype. By restoring the gut barrier function, the synbiotic treatment may impact systemic inflammation, as described in other pathological contexts (Cani *et al.*, 2009).

Second, the synbiotic approach could modulate immunity by increasing the abundance of bacterial taxa with immunomodulatory properties, such as *Clostridium* cluster XVIII, the SFB and *Lactobacillus reuteri* 100-23. Atarashi *et al.* (2013) have previously demonstrated that a selected mixture of *Clostridia* strains falling within *Clostridium* clusters IV, XIVa and XVIII was able to induce Tregs. In accordance with this result, we found a strong correlation between *Clostridium* cluster XVIII and the expression of Foxp3 in the intestine and in the spleen. The SFB have a unique ability to educate the gut immune

system and to induce a healthy state of physiological inflammation, notably through the IL-17 pathway (Ivanov *et al.*, 2009; Schnupf *et al.*, 2015). Accordingly, intestinal IL-17A expression increased in response to the synbiotic treatment. Finally, because *L. reuteri* 100-23 caused a slight increase in the numbers of Tregs in the spleen of *Lactobacillus*-free mice (Livingston *et al.*, 2010), the synbiotic-induced increase in Foxp3 expression may also be directly mediated by *L. reuteri* 100-23.

The portal metabolome reflected mainly a state of energy demand in leukaemic mice, where significantly decreased glucose and lipoproteins levels, concomitant with increased levels of creatine and lactate, were observed. Increased creatine levels are typical of muscle wasting and consistent with the cachectic model. The increased lactate level in the portal plasma of leukaemic mice was likely a consequence of lactate production by BaF cells as previously reported (Bindels *et al.*, 2012b). Succinate, an intermediate product in the Krebs cycle of endogenous and microbial origin, was also enriched in leukaemic mice. The metabolomic analysis did not highlight any portal metabolite discriminant for the synbiotic intervention. The lack of discriminant metabolites reinforces the hypothesis that the synbiotic approach provides a benefit via subtle changes in immunity or by impacting metabolic pathways that produce metabolites in low levels, such as propionate, which although detected, was below the limit of quantification in this study. Indeed, we have previously provided strong evidence to suggest that propionate produced through the bacterial fermentation of ITF reduces the proliferation of BaF cells in the liver (Bindels *et al.*, 2012b).

The present study demonstrated that cancer cachexia was associated with profound alterations of the gut microbiota in two animal models. Our data further showed that dietary modulation of the gut microbiota that mitigated some of the dysbiotic patterns restored the intestinal gut barrier and immune function, thereby reducing cancer and associated cachexia. In addition, the synbiotic approach prolonged survival, a relevant primary outcome for nutrition therapy in cancer (Baracos *et al.*, 2012). As all of the animal models have their limitations, exploration of the gut microbial ecosystem in cancer patients with cachexia is now needed before any extrapolation to humans. Which doses of synbiotics would have to be used in clinical trials to translate our findings remains highly speculative. Useful information can be obtained from published works, mainly in the context of metabolic alterations linked to obesity, which pointed out changes in the gut microbiota similar in mice and obese women upon prebiotic consumption that were associated with improvement of host metabolism (Dewulf *et al.*, 2013).

Together, these data suggest a role for the gut microbiota and the intestinal barrier in the therapeutic management of cancer and associated

cachexia. Future studies will be focused on investigating the causative role of specific gut microbes and altered intestinal homeostasis in the progression of cancer and associated cachexia, and assess the relevance of our findings in cancer patients with cachexia.

Conflict of Interest

The authors declare no conflict of interest.

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