Journal of Functional Foods 45 (2018) 223–232

Contents lists available at ScienceDirect

Journal of Functional Foods

journal homepage: www.elsevier.com/locate/jff

Prebiotic effects of white button mushroom (*Agaricus bisporus*) feeding on succinate and intestinal gluconeogenesis in C57BL/6 mice

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ARTICLE INFO

Keywords:
Mushroom
Succinate
Propionate
Microbiota
Intestinal gluconeogenesis

ABSTRACT

The mechanisms by which white button (WB) mushrooms (*Agaricus bisporus*) may influence health are unclear. WB feeding (1%) resulted in changes in the composition of microbiota in conventional (CV) mice to expand a population of *Prevotella* that produce propionate and succinate. Microbial propionate and succinate production induced expression of genes important for intestinal gluconeogenesis (IGN) via the gut-brain neural circuit. Reduced hepatic glucose production was a metabolic benefit of IGN that was found in WB fed CV mice. In the absence of microbiota or in mice with disruptions in the ability to sense microbiota there was no WB mediated effect. WB-fed lean mice had a small but significant improvement in glucose sensitivity. WB feeding resulted in shifts in the microbiota that induced IGN and improved glucose homeostasis.

1. Introduction

Edible mushrooms have been touted as low fat high fiber foods (Zou, Hoseinifar, Miandare, & Hajimoradloo, 2016). Data from the National Health and Nutrition Examination Survey showed that consuming mushroom-enriched diets reduced the risk of being overweight or obese and having metabolic syndrome (Feeney et al., 2014). People that consumed mushrooms, as a replacement for meat, were satiated, and the mushrooms improved the efficacy of weight loss intervention (Cheskin et al., 2008; Poddar et al., 2013). In rats with diabetes and hypercholesterolemia, feeding white button (WB) mushrooms (*Agaricus bisporus*) for 4 wks lowered blood glucose and cholesterol levels (Jeong et al., 2010). In the gastrointestinal (GI) tract, feeding mice diets that contained 1% WB mushrooms resulted in accelerated healing following chemical or infectious injury (Varshney et al., 2013; Yu, Weaver, Martin, & Cantorna, 2009). Consuming edible mushrooms has been shown to regulate various host physiological responses including host immunity, cancer, diabetes, and inflammatory bowel disease (Feeney et al., 2014).

Edible mushrooms contain numerous bioactive compounds including β-glucans (Cheung, 2013), dietary fiber (Cheung, 2013), ergothioneine (ERGO) (Weigand-Heller, Kris-Etherton, & Beelman, 2012), and several micronutrients (Feeney et al., 2014) including ergocalciferol (vitamin D_2), riboflavin, niacin, pantothenic acid, copper, and selenium. The contents of the various bioactive compounds vary with variety (β-glucans) (Zhu, Du, Bian, & Xu, 2015), soil (selenium and copper) (Stojkovic et al., 2014) and UV/sunshine availability (vitamin D_2) (Keegan, Lu, Bogusz, Williams, & Holick, 2013). In the US 90% of the mushrooms consumed are *Agaricus bisporus*, which includes both the WB and brown Portobello varieties. It is unclear whether a single bioactive compound is responsible for the protective effects of edible mushrooms or whether there are multiple different bioactive compounds that together make whole mushrooms beneficial for health.

Gut microbial ecology is regulated by the diet (Dalby, Ross, Walker, & Morgan, 2017; Ooi et al., 2014; Tremaroli & Backhed, 2012). For example, the prevalence of *Bacteroidetes* increased with weight loss, either by fat- or carbohydrate-restricted low-calorie diets (Ley, Turnbaugh, Klein, & Gordon, 2006). Not only do macronutrients in the diet affect the microbiota, but also deficiencies in micronutrients (vitamin D deficiency) alter the microbial communities in the gut

Abbreviations: CV, conventional; CTRL, control; ERGO, ergothioneine; FFAR3, free fatty acid receptor; GF, germfree; GI, gastrointestinal; IGN, intestinal gluconeogenesis; LEfSe, linear discriminant analysis effect size; MNA, 1-methylnicotinamide; OPLS-DA, orthogonal projection to latent structure-discriminant analysis; OTU, operational taxonomic unit; PCA, principal component analysis; PICRUSt, phylogenic investigation of communities by reconstruction of unobserved states; SCFAs, short-chain fatty acids; SGLT3, sodium glucose co-transporter; TSP, sodium 3-(trimethylsilyl) [2,2,3,3-$^2H_4$] propionate; WB, white button

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https://doi.org/10.1016/j.jff.2018.04.008
Received 19 December 2017; Received in revised form 3 April 2018; Accepted 7 April 2018

(Cantorna, McDaniel, Bora, Chen, & James, 2014; Ly, Litonjua, Gold, & Celedon, 2011). In addition, dietary fiber is indigestible by the mammalian host but readily digested by the gut microbiota (De Filippo et al., 2010; De Vadder et al., 2016; Wu et al., 2011). Alterations in the microbiota result in metabolic changes for the bacteria and the host (Musso, Gambino, & Cassader, 2011; Nicholson et al., 2012). Thus, dietary interventions are a potential tool to modulate gut microbiota and have applications in the maintenance of gastrointestinal homeostasis, prevention and treatment of chronic diseases associated with dysbiosis of the microbiota.

Feeding mice diets that contain WB mushrooms alters the microbiota in mice (Varshney et al., 2013). The shifts in the microbiota with WB feeding were associated with changes in colonic injury and recovery from colitis (Yu et al., 2009). The β-glucans found in WB mushrooms could directly stimulate toll-like receptors on immune cells (Chan, Chan, & Sze, 2009). Alternatively the microbiota might metabolize the fiber in the WB mushrooms to produce short-chain fatty acids (SCFAs) and other metabolites with biological function (Feeney et al., 2014; Wong, Wong, Kwan, & Cheung, 2005). The beneficial effects of WB mushrooms could be direct effects on the host and/or indirect effects on the microbiota to regulate the host or both types of effects. WB mushrooms contain numerous potentially bioactive components that could regulate host and/or microbial metabolism.

Edible WB mushrooms have been shown to improve immune function, improve weight loss, and improve healing in the gastrointestinal tract (Feeney et al., 2014). Some of the benefits of WB feeding may be due to their impact on the microbiota (Varshney et al., 2013). The aims of the study were to determine the effects of WB feeding on host versus microbial metabolism. Based on the metabolomics analyses (microbially produced succinate and propionate), additional experiments explored the potential down-stream effects of WB feeding on glucose homeostasis.

2. Materials and methods

2.1. Mice and diets

C57BL/6 wild type mice were originally from Jackson Laboratories (Bar Harbor, MN) and bred at the Pennsylvania State University (University Park, PA). C57BL/6 MyD88 -/- mice were a gift from Dr. Matam Vijay Kumar (Pennsylvania State University). Germfree (GF) mice were from the Pennsylvania State University Gnotobiotic Facility. Animal experiments were performed using protocols approved by the Pennsylvania State University Institutional Animal Care and Use Committee.

Agaricus bisporous WB mushrooms were a gift from Giorgio Foods (Temple, PA). The mushrooms were freeze-dried and ground into a fine powder and sent to Teklad Diets (Madison, WI) to be incorporated into the control (CTRL) TD 89124 diet at 1 g/100 g (1%). Mice were fed the weight equivalent of 1 human serving of whole WB mushrooms which equals 75–100 g fresh WB weight in a human diet (Chan et al., 2009). The WB and CTRL diets were irradiated to sterilize them for use with GF mice. Six wks old male mice (n = 12) were randomly divided into two groups. Half of the mice were fed CTRL diets and the other half of the mice were fed WB diets for 2 or 7 wks. CV mice were housed in the same animal room at the Pennsylvania State University. GF status was monitored continuously and confirmed for the mice at sacrifice.

2.2. Histopathology and clinical biochemistry

The formalin-fixed liver tissues were embedded in paraffin wax, sectioned (3–5 μm) and stained with hematoxylin and eosin. Sections were evaluated blinded by a board certified pathologist. Serum alanine transaminase and alkaline phosphatase were measured using the VetScan VS2 and the Mammalian Liver Profile rotor (Abaxis Inc., Union City, CA) according to the manufacturer's instructions.

2.3. Glucose tolerance test

Glucose tolerance was done as previously described and following a 14–15 h overnight fast (Ayala et al., 2010). Briefly, glucose tolerance test was measured following intraperitoneal injection of 20% glucose (2.0 g/kg body weight) in PBS and blood glucose was measured after glucose injection. Blood glucose concentrations were measured using OneTouch UltraMini blood glucose monitor (LifeScan, Inc., Milpitas, CA).

2.4. ERGO in tissues by UPLC-TQS-MS

ERGO was measured in tissues by an Acquity UPLC system coupled to a Waters Xevo TQS MS with a C18 BEH (2.1 × 100 mm, 1.7 μm) UPLC column (all waters, Milford, MA) using the methods of Noritaka and Wi (Lee, Park, Ahn, & Ka, 2009; Nakamichi et al., 2016), with minor modifications. ERGO standard was obtained from Sigma-Aldrich Inc. (St. Louis, MO). Briefly, 25–30 mg of liver or kidney tissue from CV or GF mice was homogenized in 150 μl HPLC water. Samples were deproteinated with two volumes of acetonitrile. Following centrifugation, 250 μl of the supernatant was transferred to an autosampler vial. Analytes were detected by multiple reaction monitoring and quantified using the standard curve with concentrations ranging from 0.1 μg/ml to 50 μg/ml.

2.5. Urine microbial metabolite profiling by UPLC-QTOF-MS

Global analysis of urine samples was performed by reverse phase UHPLC using a Prominence 20 UFLCXR system (Shimadzu, Columbia, MD) with a waters (Milford, MA) CSH C18 column (2.1 × 100 mm × 1.7 μm particle size). Briefly, 10 μl of urine was mixed with 50 μl of ice cold methanol. The samples were incubated at −20 °C for one hour after vortexing. Following centrifugation at max speed for 20 min, the supernatants were transferred to an autosampler vial. Peak alignment, deconvolution, and normalization were performed by PeakView (Sciex, Concord, Canada). The identification of urine microbial metabolites was performed using the published results (Varshney et al., 2013) (Supplementary Table S1).

2.6. Jejunum intestinal gluconeogenesis metabolite profiling by UPLC-Orbitrap-MS

Targeted analysis of jejunum samples was performed by LC-MS using a modified version of an ion pairing reversed phase negative ion electrospray ionization method (Lu et al., 2010). Jejunum tissues (~30 mg) were extracted twice with 500 μl of ice cold methanol (80% v/v). The combined supernatant (1 ml) was dried *in vacum* and reconstituted in 100 μl of methanol (3% v/v). The LC-MS system consisted of a Dionex Ultimate 3000 quaternary HPLC pump, a Dionex 3000 column compartment, a Dionex 3000 autosampler, and an Exactive plus orbitrap mass spectrometer controlled by Xcalibur 2.2 software (all from Thermo Fisher Scientific, Waltham, MA). Analytes of succinate, pyruvate, fructose-1,6-bisphosphate, fructose-6-phosphate, and glucose-6-phosphate were identified by software package MZmine 2 (Pluskal, Castillo, Villar-Briones, & Oresic, 2010) with an in-house database.

2.7. ^{1}H NMR-based metabolomics experiments

Dried mushroom powder (~ 100 mg) was homogenized with 600 μl PBS (0.1 M, pH = 7.43, 50% v/v D_2O) containing 0.005% sodium 3-(trimethylsilyl) [2,2,3,3-$^{2}H_4$] propionate (TSP) and centrifuged to obtain the supernatant. Biological sample preparation for NMR analyses were performed as previously described (Shi, Xiao, Wang, & Tang, 2013).

All ^{1}H NMR spectra were acquired at 298 K on a Bruker AVIII

600 MHz NMR spectrometer with an inverse cryogenic probe (Bruker Biospin, Rheinstetten, Germany). Spectrum was acquired using the first increment of the NOESY pulse sequence (NOESYPR1D). To facilitate NMR signal assignments, a set of two-dimensional (2D) NMR spectra (for more detailed methods, see Supplementary files "^{1}H NMR Spectroscopy"). All ^{1}H NMR spectra were phase- and baseline-corrected manually using TOPSPIN (V3.0, Bruker Biospin) and referenced to TSP (δ 0.00). All the spectra were integrated into regions (δ 0.50–10.00) with equal bucket-width of 0.004 ppm (2.4 Hz) using the AMIX package (V3.8, Bruker Biospin). The ^{1}H NMR spectra of the WB powder is shown in Supplementary Fig. S1. The ^{1}H NMR spectra of cecal content (cecum) and liver from mice fed CTRL or WB are shown in Supplementary Fig. S2. Metabolite assignments were carried out on the basis of published results (Dong, Zhang, Hao, Tang, & Wang, 2013; Tian, Zhang, Wang, & Tang, 2012).

Multivariate data analyses, including principal component analysis (PCA) and orthogonal projection to latent structure-discriminant analysis (OPLS-DA) were carried out using the SIMCA-P+ software (Version 13.0, Umetrics, Umea, Sweden). The quality of the model was described by the parameters R^2X and Q^2. The validity of the OPLS-DA model was further assessed with CV-ANOVA tests for significance and $p < 0.05$ (Eriksson, Trygg, & Wold, 2008). Back-transformed loadings from the OPLS-DA models were performed with color-coded correlation coefficient for variables, using an in-house developed script for MATLAB (The Mathworks Inc., Natwick, MA). The significance of the metabolite contribution to the group separation was indicated by the color-coded correlation coefficient, with a "hot" color (e.g., red) being more significant than a "cold" color (e.g., blue). In this study, cut off values of $|r| > 0.754$ were regarded as important ones on the basis of the discrimination significance ($p < 0.05$) (for more detailed methods, see Supplementary files "NMR data processing and multivariate data analysis").

2.8. Tissue RNA isolation and quantitative real-time PCR

Total RNA was isolated from intestine tissues including jejunum and colon (~40 mg) by TRIzol reagent (Invitrogen, Carlsbad, CA) according to the manufacturer's protocol. The concentration of RNA was determined by NanoDrop (ND-1000, V 3.3). cDNA was synthesized from 1 μg of total RNA using qScript cDNA SuperMix (Quanta Biosciences, Gaithersburg, MD). Quantitative PCR (qPCR) reactions were performed using SYBR green QPCR master mix with an ABI Prism 7900HT Fast real-time PCR sequence detection system (Applied Biosystems, Waltham, MA). The primers used in this study were listed in Supplementary Table S2. qPCR conditions were 40 cycles of 95 °C for 20 s, 95 °C for 0.01 s, 60 °C for 20 s, 95 °C for 15 s, 60 °C for 15 s, and 95 °C for 15 s. Gene express data were normalized to β-*actin* mRNA levels using $\Delta\Delta C_T$ method.

2.9. Gut microbiota analysis

DNA from cecal contents were extracted using E.Z.N.A. ® stool DNA kit (Omega Bio-Tek Inc., Norcross, GA) according to the manufacturer's instructions. Targeted bacteria were analyzed by qPCR using primers targeted at 16S ribosomal DNA of *Firmicutes*, *Bacteroidetes*, *Actinobacteria*, γ-*Proteobacteria*, and *Prevotella*. *Prevotella copri* is an important succinate producer in the intestine (De Vadder et al., 2016). The primers used in this study are listed in Supplementary Table S3. qPCR assays were carried out using SYBR Green qPCR Master Mix on an ABI Prism 7900HT Fast Real-Time PCR sequence detection system. The results were normalized to 16S ribosomal (universal) DNA sequences and expressed as the relative difference compared to CTRL using the $\Delta\Delta C_T$ method.

The extracted bacterial DNA from cecal contents was amplified using the V4V4 primer set (515F and 806R). Product verification was done using 1% agarose gel electrophoresis and a DNA 7500LabChip on the Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clare, CA). PCR products were sent to the Penn State Genomics Core Facility (University Park, PA) for library preparation. 250 × 250 paired end sequencing was performed on the Illumina Miseq platform. 16S rRNA gene amplicon sequence results were analyzed using the MOTHUR platform (Kozich, Westcott, Baxter, Highlander, & Schloss, 2013) and were aligned to the SILVA and the Green-Genes-databases for separate analyses. Alignment to the SILVA database provided the data for the GUnifrac analysis. A phylogenic tree and operational taxonomic unit (OTU) table were obtained from the Mothur Bayesian classifier (Kozich et al., 2013) and uploaded to R studio for GUnifrac analysis (Chen et al., 2012). After Green Genes alignment, a .biom file was created and uploaded onto the Huttenhower galaxy page. PICRUSt (Phylogenic Investigation of Communities by Reconstruction of Unobserved States) analysis was done on the biom file resulting in a pathway abundance file (Langille et al., 2013). LEfSe (Linear discriminant analysis Effect Size) was used to obtain statistically significant and biologically relevant pathways from pathway abundance file (Segata et al., 2011). All data have been deposited in NCBI's Sequence Read Archive under the accession number PRJNA418167.

2.10. Statistics

Values are the median and interquartile range. Graphical illustrations and statistical analysis were performed using GraphPad Prism (v 6.0, GraphPad). The data was analyzed using the unpaired, non-parametric Mann-Whitney test and $p < 0.05$ were considered as significant.

3. Results

3.1. Higher ERGO levels in tissues from WB-fed mice

As expected and confirming the delivery of WB mushrooms in the WB-fed mice, ERGO levels were higher in both the liver and kidney of WB-fed CV (Fig. 1A) and GF (Fig. 1B) mice. The WB feeding had no effect on weight gain or body weight of the mice compared to control (CTRL) feeding (Supplementary Figs. S3A-B). The liver histopathology was normal in WB-fed and CTRL-fed mice (Supplementary Fig. S3C). In addition, the serum alanine transaminase and alkaline phosphatase levels were the same in WB and CTRL-fed mice (Supplementary Fig. S3D). WB feeding for 2 wks resulted in higher ERGO levels but no differences in liver histopathology or serum alanine transaminase or alkaline phosphatase levels.

3.2. Microbial induced succinate and propionate following WB feeding of CV mice

Pairwise OPLS-DA was done comparing the data for the cecal contents of CV (top) and GF (bottom) WB and CTRL fed mice (Fig. 2A). The model quality indicators showed that metabolites in the cecum were distinctive in both CV and GF mice after 2 wks of WB feeding (Fig. 2A and Supplementary Table S4). The NMR spectra in the WB fed CV and GF mice were completely different indicating microbial metabolism of the WB mushrooms (Supplementary Figs. S2A-B). GF mice fed WB mushrooms had significantly higher mannitol in the cecal contents compared to the CTRL fed GF mice (Fig. 2A). Mannitol was found in the freeze dried WB mushrooms (Supplementary Fig. S1) and confirmed that mannitol is the major soluble sugar in the WB diets (Lo, Chien, Mishchuk, Slupsky, & Mau, 2016). GF mice were unable to ferment the mannitol in the WB mushrooms (Fig. 2A). Conversely, the mannitol peaks were missing in the WB-fed CV mice suggesting microbial fermentation of the mannitol in the CV mice (Fig. 2A). In addition, the WB fed CV mice had significantly higher levels of several nucleotide metabolites (e.g., uracil, xanthine, and hypoxanthine) and carboxylic acids (propionate and succinate, Fig. 2A-B). WB feeding of CV mice resulted in decreased levels of branched chain amino acids (leucine, isoleucine,

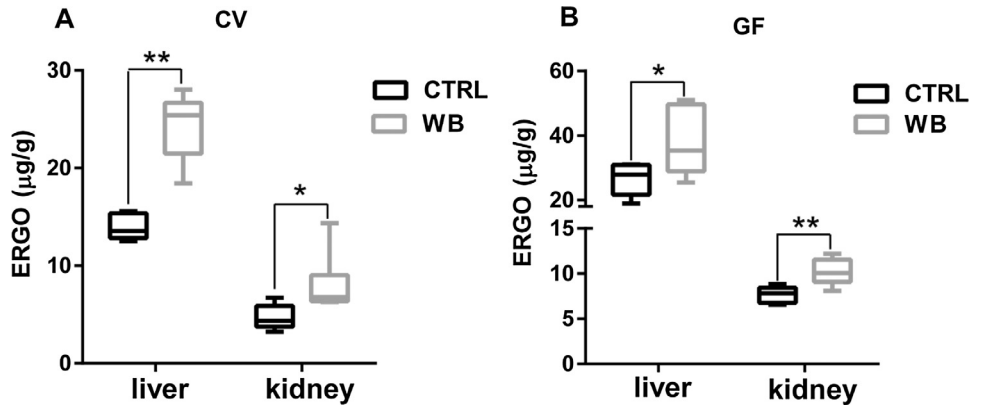

Fig. 1. Higher ERGO levels in tissues from WB-fed mice. ERGO levels in the liver and kidney tissues were measured by UPLC-TQS-MS in CV (A) and GF (B) mice. Values are the median and interquartile ranges of n = 6 mice per group. $^{*}p < 0.05$, $^{**}p < 0.01$.

and valine), alanine, and trimethylamine in the cecum (Fig. 2A). The increases in propionate and succinate with WB feeding happened only in CV mice and there was no cecal succinate or propionate peak in the GF spectra (Fig. 2A and Supplementary Fig. S2B). Mice with disruptions in microbial signaling (MyD88-/-) failed to show an increase in propionate and succinate with WB feeding (Fig. 2C). Previously, higher levels of hippuric acid, cinnamoylglycine, and dopamine glucuronide in the urine were shown following 6 wks of WB-feeding (Varshney et al., 2013). Using a different diet and shorter 2 wks WB feeding, induced an increase in cinnamoylglycine and dopamine glucuronide but no change in hippuric acid in the urine of WB-fed mice (Supplementary Fig. S4). In GF mice fed WB mushrooms there were significant decreases in lactate, choline, and amino acids including branched chain amino acids, tyrosine, and phenylalanine in the cecum compared to CTRL fed GF mice

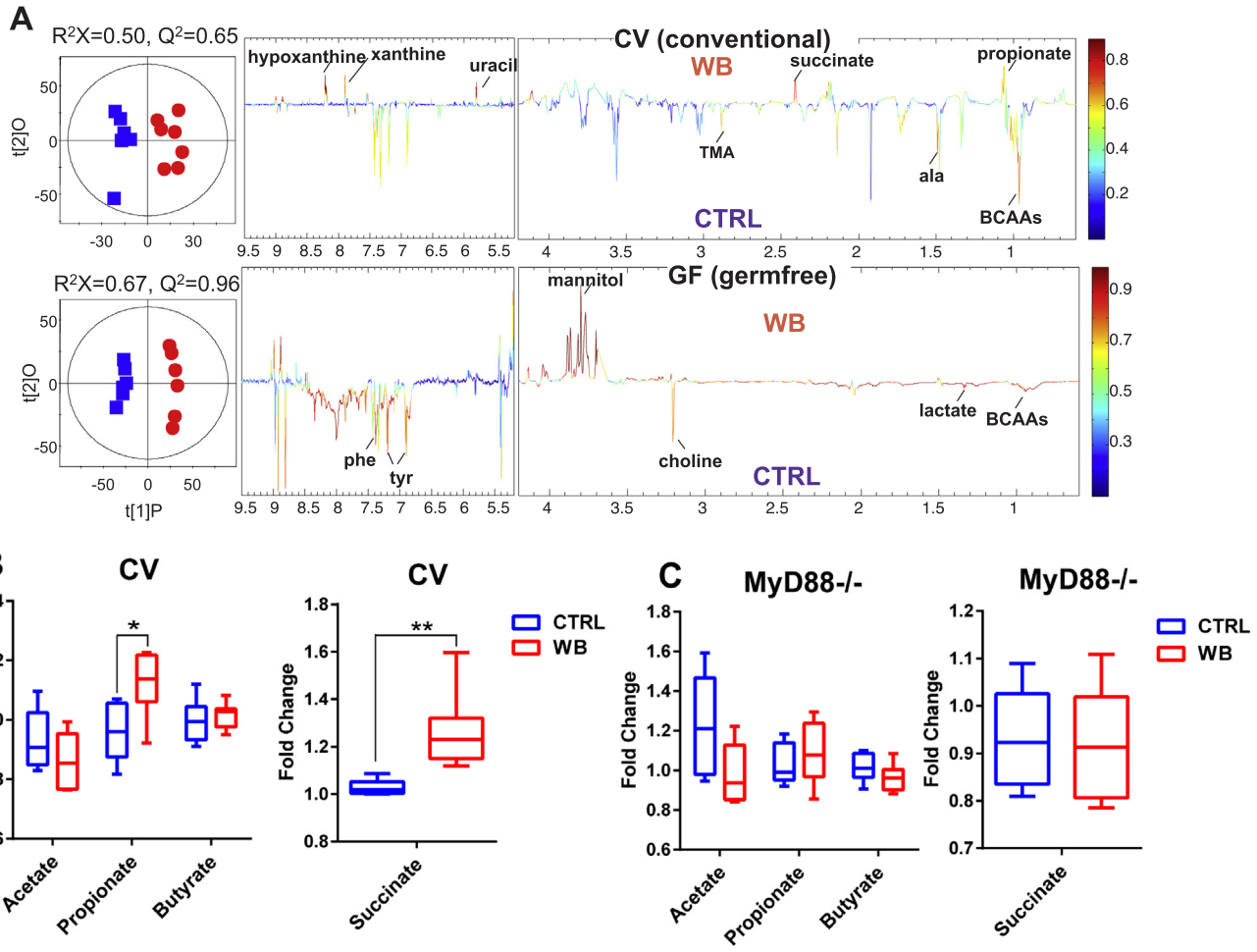

Fig. 2. Microbial induced succinate and propionate following WB feeding of CV mice. (A) OPLS-DA scores plots (left) and coefficient plots (right) derived from ^{1}H NMR spectra of the cecal content of CV and GF mice after 2 wks of (■) CTRL or (●) WB feeding. Models were evaluated with CV-ANOVA and showed significant effects of WB feeding; (CV) $p = 0.045$ and (GF) $p = 5.8E-5$. (B-C) Relative abundance of cecal microbial metabolites including SCFAs and succinate measured by ^{1}H NMR data of (B) CV and (C) MyD88-/- mice after 2 wks of CTRL or WB feeding. Values are the median and interquartile ranges of n = 6 per group. $^{*}p < 0.05$, $^{**}p < 0.01$. Abbreviations: BCAAs, branched chain amino acids; ala, alanine; TMA, trimethylamine; tyr, tyrosine; phe, phenylalanine.

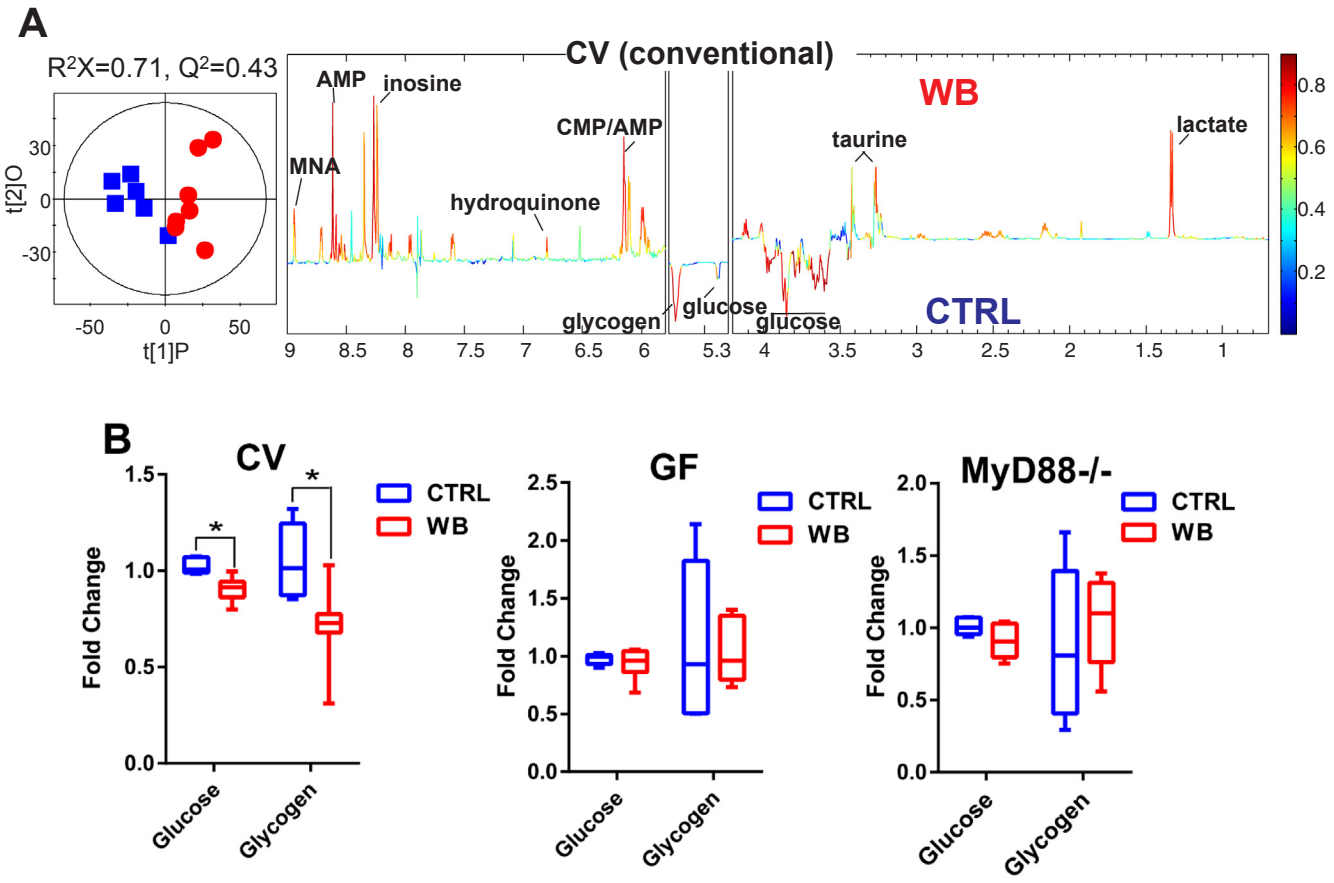

Fig. 3. Reduced liver glucose and glycogen with WB feeding of CV mice. (A) OPLS-DA scores plot (left) and coefficient plot (right) derived from ^{1}H NMR spectra of liver samples from CV mice after 2 wks of (■) CTRL or (●) WB feeding. The models was evaluated with CV-ANOVA with $p = 0.021$. (B) Relative abundance of liver glucose and glycogen measured by ^{1}H NMR data from CV, GF, and MyD88-/- mice after 2 wks of CTRL or WB feeding. Values are the median and interquartile ranges of n = 6 per group. $^{*}p < 0.05$. Abbreviations: CMP, cytidine 5′-monophosphate; AMP, adenosine monophosphate; MNA, 1-Methylnicotinamide.

(Fig. 2A). The effect of WB-feeding on lactate, choline, tyrosine, and phenylalanine did not occur in CV mice. The reduction in branch chain amino acids happened in both WB-fed CV and GF mice. Microbial fermentation of the mannitol in the WB mushrooms resulted in induction of propionate and succinate in the cecum of the WB fed mice.

3.3. Reduced liver glucose and glycogen with WB feeding of CV mice

Pairwise OPLS-DA indicators showed significant metabolic changes in the liver of CV mice after WB feeding but no differences in the liver metabolites from WB- and CTRL-fed GF mice (Fig. 3A and Supplementary Table S4). Feeding WB mushroom to CV mice significantly elevated the levels of lactate, taurine, inosine, hydroquinone, cytidine 5′-monophosphate, adenosine monophosphate, and 1-methylnicotinamide and significantly decreased the levels of liver glucose and glycogen in CV mice (Fig. 3A). The changes in liver glucose and glycogen with WB feeding did not occur in either GF or MyD88-/- mice (Fig. 3B). Glucose tolerance tests showed no effect of WB feeding after 2 wks (Supplementary Fig. S5A) but a small but significantly lower peak glucose level in the glucose tolerance test after 7 wks of WB feeding (Supplementary Fig. S5B). WB feeding of CV mice resulted in reduced levels of glucose and glycogen in the liver and small but significant improvements in glucose sensitivity.

3.4. Alterations in the microbial community structure with WB feeding

Previous work showed that WB feeding (1% diet) resulted in changes to the fecal microbiota that were evident at 2 wks and stabilized after 4 wks of WB feeding (Varshney et al., 2013). Diets were not the same as the previous study (Varshney et al., 2013) since irradiated commercial diets were needed for GF mice. Generalized Unifrac analysis of 16S rRNA gene sequencing of the cecal contents from CV mice fed WB or CTRL diets for 2 wks resulted in significantly different microbes in the two diet groups (Fig. 4A). WB fed mice had significantly higher numbers of *Bacteroidetes* phyla members (Fig. 4B and Supplementary Fig. 6). WB feeding also resulted in higher numbers of bacteria from the class *Epsilonproteobacteria* and *Bacteroidia*, order *Campylobacterales*, *Lactobacillales*, and *Bacteroidales*, and family *Helicobacteraceae*, *Clostridium_XIV*, *Lactobacillaceae*, *Porphyromonadacea*, *Planococcaceae*, and *Prevotellaceae*, genus *Helicobacter*, *Lactobacillus*, *Bacilli*, and *Coprobacillus* in WB-fed mice (Fig. 4B). WB feeding decreased the numbers of the families *Pseudoflavonifractor*, *Dorea*, *Clostridium_XI*, and *Peptostreptococcaceae* (Fig. 4B). There was a significant increase in the numbers of *Prevotella* (Fig. 4C) in the cecum of WB fed mice. The *Prevotella* are known to be succinate producers and the increase in *Prevotella* corresponded with the increase in succinate following WB feeding (Fig. 4C and 2B). WB feeding in the MyD88-/- mice failed to induce *Prevotella* or succinate production (Fig. 4D and 2C). PICRUSt was performed on the 16S rRNA gene sequencing data to predict significant bacterial gene expression changes that were affected by WB feeding using LEfSe (Fig. 4E). The data predicts that the bacteria from the WB-fed mice were enriched for bacterial genes that encode purine and pyrimidine metabolism, amino acid metabolism, glycan degradation, and carbohydrate metabolism and lower levels of genes for signal transduction compared to the bacterial genes expressed in the CTRL-fed mice (Fig. 4E). WB feeding resulted in alterations in the types of

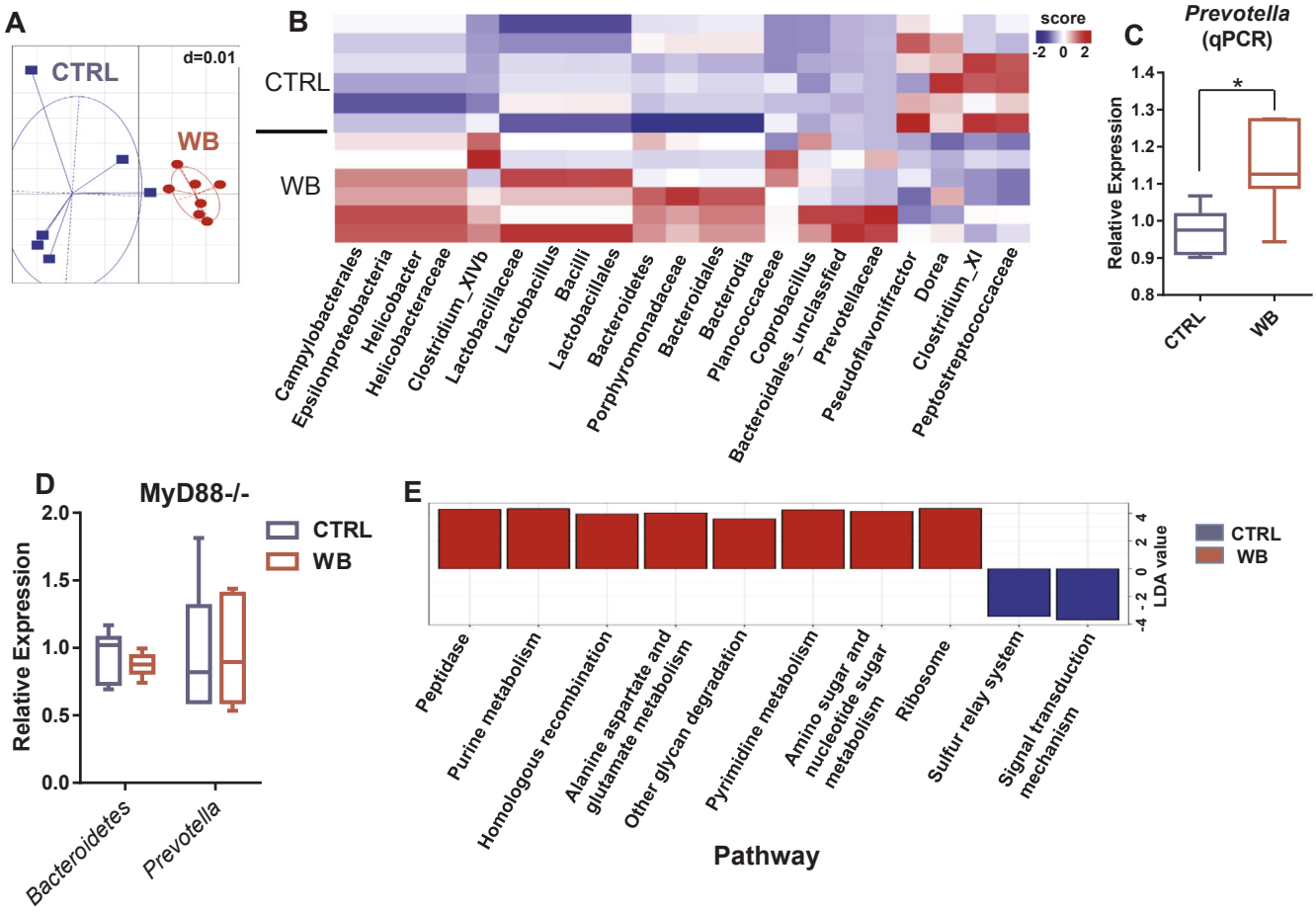

Fig. 4. Alteration of microbial community structure in WB-fed CV mice. (A) Generalized Unifrac analysis of the total population of the gut microbiome in the cecum of CV mice after 2 wks (■) CTRL or (●) WB feeding. (B) Heat map representation of the abundance of microbiota that were significantly increased (red) or decreased (blue) following WB-feeding as compared to CTRL-feeding. (C) qPCR analysis of *Prevotella* in the cecal contents from CV mice after 2 wks CTRL or WB feeding. (D) qPCR analysis of *Bacteroidetes* and *Prevotella* in the cecal contents from MyD88-/- mice after 2 wks CTRL or WB feeding. (E) PICRUSt analysis results of predicted functional pathways in the gut microbiota. The pathway abundance values for (blue) CTRL and (red) WB are representative of the amount of genes expressed in a particular pathway from each sample. These pathways were also ordered by decreasing coverage, which was calculated based on the total possible amount of genes (according to the Metacyc database). All pathways shown are significant according to LEfSe. LEfSe uses the Kruskal-Wallis test and also the Wilcoxon test at a cutoff of 0.05 to determine significant and biologically relevant pathways between two groups. Values are the median and interquartile ranges of n = 6 per group. $^{*}p < 0.05$.

bacteria found in the cecum and changes in bacterial gene expression compared to the bacteria and genes expressed in CTRL-fed mice.

3.5. Increased G6pase, Glut2, and Pepck mRNA and fructose-1-6-bisphosphate, fructose-6-phosphate, and glucose-6-phosphate metabolites in WB-fed mice

Recent evidence suggests that microbial succinate improves glucose homeostasis via IGN (De Vadder et al., 2016). CV mice fed WB diets had higher levels of fructose-1-6-bisphosphate, fructose-6-phosphate, and glucose-6-phosphate in the jejunum than CTRL-fed mice (Fig. 5A). WB feeding had no effect on these same metabolites in GF mice (Fig. 5A). Dietary WB feeding increased mRNAs for *G6pase*, *Glut2*, and *Pepck* in the jejunum of CV mice but not GF mice (Fig. 5B). WB feeding in GF mice significantly decreased mRNA for *G6pase* and *Glut2* in the jejunum compared to CTRL-fed mice (Fig. 5B). The mRNA expression for a portal glucose sensor, sodium glucose co-transporter (SGLT3) that initiates a neural gut-brain response (Mithieux, 2014b; Soty et al., 2015) was measured in the jejunum and colon. WB feeding resulted in significant increases in mRNA for *Sglt3a* in jejunum and *Sglt3b* in the colon from CV mice but not GF mice (Fig. 5B-C). The mRNA expression for free fatty acid receptor (FFAR3), which is a SCFA receptor, also showed a significant elevation in colon tissues from WB fed CV mice but not in GF mice (Fig. 5C). WB feeding resulted in microbial-mediated induction of several genes and metabolites associated with IGN in CV mice.

4. Discussion

WB feeding had microbial dependent and microbial independent effects on the metabolic profile in mice. ERGO is a natural sulfur containing amino acid found in WB mushrooms that functions as an antioxidant (Weigand-Heller et al., 2012). WB feeding resulted in increased ERGO levels that were independent of the microbiota. Conversely, WB feeding resulted in induction of cecal propionate and succinate by microbial fermentation of mannitol in WB mushroom. Mannitol is the major soluble sugar in freshly harvested mushroom fruiting bodies (Lo et al., 2016; Tseng & Mau, 1999). Feeding mannitol to pigs and rats resulted in the fermentation of mannitol to produce more SCFAs including both butyrate and propionate (Maekawa et al., 2005; Orishita, 1994). Succinate and propionate are common end products of sugar/lactate fermentation by the microbiota in the gut (Hosseini, Grootaert, Verstraete, & Van de Wiele, 2011). Propionate is produced by the microbial succinate pathway (Reichardt et al., 2014). Here the higher levels of cecal succinate and propionate corresponded to the

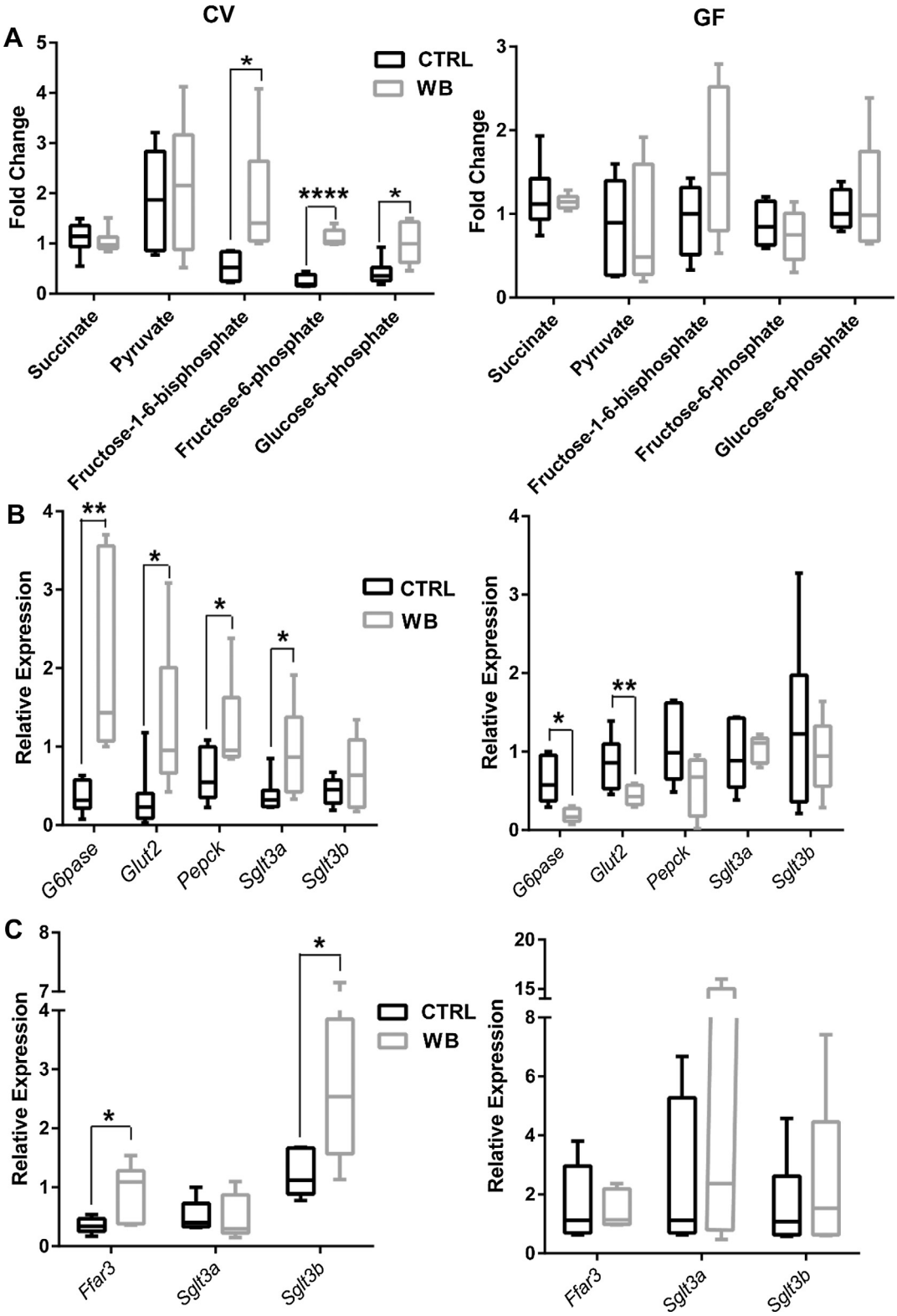

Fig. 5. WB feeding induced IGN and gut-brain neural circuits in CV mice. Mice were fed CTRL or WB diets for 2 wks. (A) Relative abundance of IGN metabolites measured by UPLC-Orbitrap-MS in the jejunum from CV and GF mice. (B) qPCR analysis of mRNA levels of *G6pase*, *Glut2*, *Pepck*, *Sglt3a*, and *Sglt3b* in the jejunum from CV and GF mice. (C) qPCR analysis of mRNA levels of *Ffar3*, *Sglt3a*, and *Sglt3b* in the colon from CV and GF mice. Values are the median and interquartile ranges of n = 6 per group. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, $^{****}p < 0.0001$.

disappearance of mannitol in the WB-fed CV mice, suggesting that bacterial fermentation of mannitol was the likely source of increased SCFAs. Propionate has metabolic benefits on body weight and glucose control by activating IGN (Hosseini et al., 2011). The free fatty acid receptor FFAR3, a G-protein-coupled receptor activated by microbiota-produced SCFAs, is important in the propionate-mediated induction of IGN (De Vadder et al., 2014). Propionate is an endogenous FFAR3 ligand (Brown et al., 2003). WB feeding induced *Ffar3* mRNA in the

colon of CV but not GF mice. Feeding WB mushrooms to normal weight mice for 2–7 wks had no effect on body weight (data not shown). However, there was a small and significant improvement in glucose tolerance after 7 wks of WB feeding (Supplementary Fig. 5), even in the absence of diet-induced obesity. The decreased liver glucose and glycogen levels, increase in propionate, FFAR3 and multiple other genes and metabolites with WB feeding of CV mice show that glucose homeostasis may be improved with WB feeding (Fig. 6).

Confirming previous data that fed WB mushrooms to mice for longer and using a different diet, WB feeding resulted in alteration of microbial community structure (Varshney et al., 2013). WB-fed mice had higher representation of phylum *Bacteroidetes*, especially the *Prevotella* subtype. This increase in the *Bacteroidetes* phyla has been reported with WB feeding previously (Varshney et al., 2013) and can be used to confirm the taxonomic changes observed in this study. *Bacteroidetes* are important producers of propionate (Chen et al., 2017; Gorvitovskaia, Holmes, & Huse, 2016). *Prevotella* are known to degrade cellulose and xylans, and have been demonstrated to produce succinate in the cecum (De Filippo et al., 2010; De Vadder et al., 2016; Wu et al., 2011). The finding that MyD88 -/- mice did not increase succinate or *Prevotella* with WB feeding suggests that; either the immune toll like receptor dysfunction, and/or differences in the microbial community between MyD88 -/- and WT mice, is critical for the WB mediated effect. Other microbial effects of WB feeding include increases in *Lactobacilli* that have been shown to increase antioxidative molecules (Guo et al., 2004; Vamanu, Pelinescu, Avram, & Nita, 2013). The bacterial communities from the WB fed CV mice were predicted to have enrichment of genes important in purine and pyrimidine metabolism, amino acid metabolism, glycan degradation, and carbohydrate metabolism. The key nutrients involved in those pathway are readily provided in the WB powder (Supplementary Fig. S1). WB feeding of mice for 2 wks increased the *Prevotella* and *Bacteroidetes* bacteria and altered microbial gene expression.

WB feeding promoted IGN and reduced hepatic glucose production (Fig. 6). IGN is a mandatory function for the healthy neural control of glucose and energy homeostasis, which can be promoted by succinate and SCFAs generated by fermentation (De Vadder et al., 2014; De Vadder et al., 2016). Microbial succinate regulates IGN through several metabolites (fructose-1–6-bisphosphate, fructose-6-phosphate, and glucose-6-phosphate) and genes (*G6pase*, *Glut2*, and *Pepck*) expressed in the jejunum (De Vadder et al., 2014; De Vadder et al., 2016). Mice with an intestinal deletion in G6pase and deficient in IGN did not show metabolic benefits (weight loss and glucose control) due to SCFAs or dietary fiber (De Vadder et al., 2014). Here *G6pase*, *Glut2*, and *Pepck* mRNAs in the small intestine as well as IGN metabolites were higher following WB feeding of CV mice (Fig. 6). The portal glucose sensor SGLT3 transmits signals to the brain, and has been shown to activate control of food intake (Mithieux, 2014a). The significant increase in intestinal expression of *Sglt3* mRNA with WB feeding supports a potential effect of WB feeding on control of food intake. Suppression of hepatic glucose production is one of the strategies being targeted for diabetes therapies (Abdul-Wahed et al., 2014; C, DA, J, & AJ, 2005). Collectively, the data suggest that WB feeding has microbial mediated metabolic benefits to the host that would result in improved glucose homeostasis.

There are several limitations of the current study. The effects of the WB feeding were only tested using raw mushrooms. Cooking could alter the bioactive components in mushrooms. However, the fermentation of mannitol and fiber by the microbiota within the mushrooms should not be affected by cooking. Therefore, the main effects of the WB feeding demonstrated here that include induction of succinate and propionate and the down-stream effects of the WB feeding on IGN should not be affected by cooking. In addition, the use of mice as a model may not accurately predict the effects of WB feeding in humans. However, mice

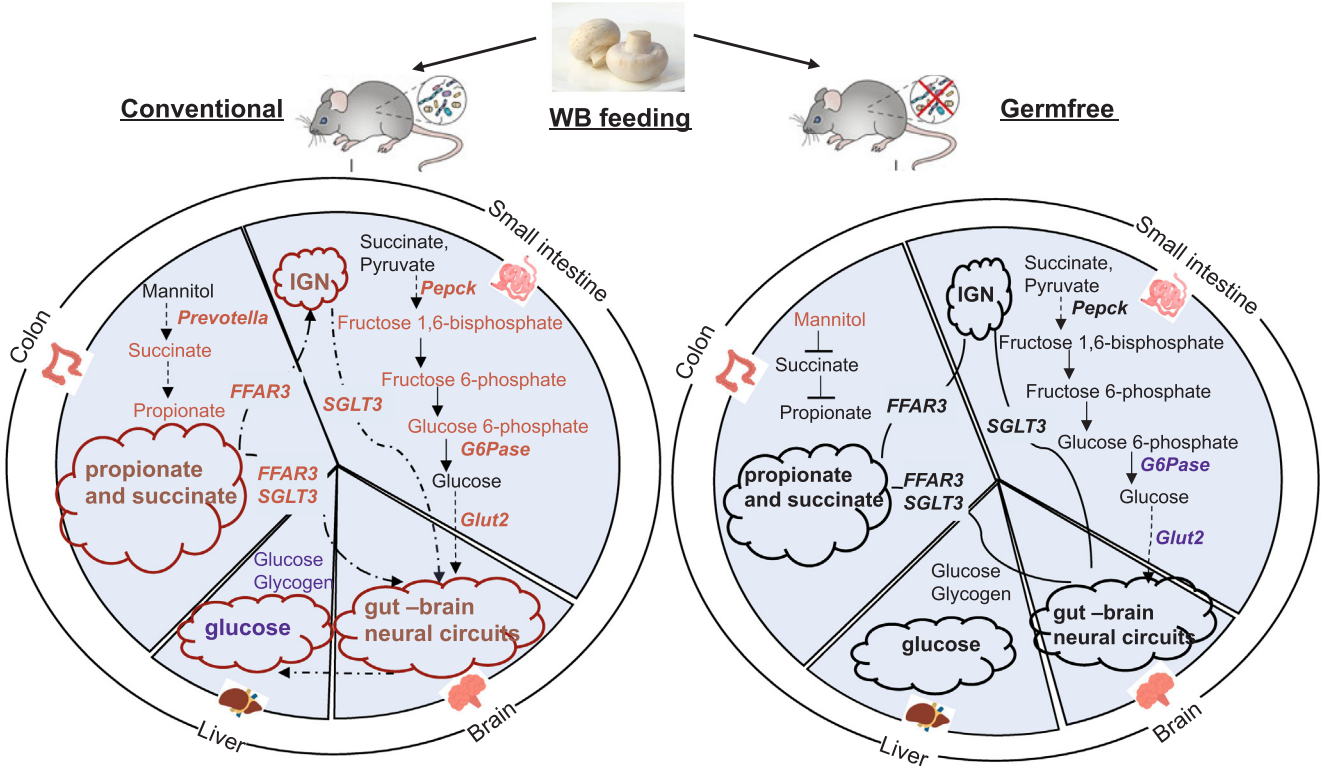

Fig. 6. Model of the metabolic effect of WB feeding. The metabolites, bacteria or mRNAs in red are higher and the metabolites or mRNAs in blue are lower in WB-fed compared to CTRL-fed mice. The WB mediated effects were predominately found only in CV mice (left panel). WB fed GF mice had higher mannitol and lower *G6Pase* and *Glut2* levels. WB feeding of CV mice induced propionate and succinate production that was associated with lower glucose and glycogen in the liver, and induction of genes important for IGN and gut/brain communication. The predominate effects of WB feeding was due to the microbial shifts that induced changes to bacterial and host metabolism.

and, in particular, gnotobiotic mice have been useful to understand the mechanisms by which diet mediated changes in the microbiota participate in glucose homeostasis (De Vadder et al., 2016; Sung et al., 2017; Zhang et al., 2017). Future work is needed to determine whether human consumption of WB mushrooms would be beneficial for glucose regulation.

5. Conclusions

WB feeding has prebiotic effects on both host and bacterial metabolism. WB feeding induced shifts in the microbiota that included expansion of *Bacteroidetes* phyla members specifically the *Prevotella* bacteria that are known to be a source of propionate and succinate. GF mice and MyD88-/- mice did not increase *Prevotella*, propionate or succinate with WB feeding. The microbially derived propionate and succinate was associated with increased IGN and increased expression of genes important in the gut-brain axis linked to satiety and hepatic glucose output. The data suggest that daily consumption of the equivalent of one serving of WB mushrooms per day could be a simple way to improve diet quality and the microbiota that would improve metabolic fitness and the development of diabetes.

Acknowledgments

We would like to thank Joselyn Allen, Nelson Thomas Peterson, and Anitha Vijay for technical help and K. Sandeep Prabhu for critical and lively discussion of the data. We thank the Giorgio Food company for providing the mushrooms used in this project.

Funding sources

The research was supported in part by the Mushroom Council to A.D.P. and M.T.C. and an American Association of Immunologists fellowship to Y.T. Additional support from the National Institutes of Health grants R56AI114972 and R01AT005378 to M.T.C.

Competing interests

The authors declare no competing financial interests.

Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.jff.2018.04.008.

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