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Metabolomic and Transcriptomic Correlative Analyses in Germ-Free Mice Link *Lacticaseibacillus rhamnosus* GG-Associated Metabolites to Host Intestinal Fatty Acid Metabolism and β-Oxidation

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Abstract

Intestinal microbiota confers susceptibility to diet-induced obesity, yet many probiotic species that synthesize tryptophan (trp) actually attenuate this effect, although the underlying mechanisms are unclear. We monocolonized germ-free mice with a widely consumed probiotic *Lacticaseibacillus rhamnosus* GG (LGG) under trp-free or -sufficient dietary conditions. We obtained untargeted metabolomics from the mouse feces and serum using liquid chromatography–mass spectrometry and obtained intestinal transcriptomic profiles via bulk-RNA sequencing. When comparing LGG-monocolonized mice with germ-free mice, we found a synergy between LGG and dietary trp in markedly promoting the transcriptome of fatty acid metabolism and β-oxidation. Upregulation was specific and was not observed in transcriptomes of trp-fed conventional mice and mice monocolonized with *Ruminococcus gnavus*. Metabolomics showed that fecal and serum metabolites were also modified by LGG-host-trp interaction. We developed an R-Script-

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Author Contributions

R.P.F. and N.G. conceptualized the study and designed experiments. W.V.L. developed the METRCA methodology. P.S. and J.F. conducted the experiments. P.S., R.P.F., J.A., J.M.A., J.F., A.J., W.V.L., and X.S. analyzed the data, and R.P.F. wrote the manuscript; and N.G., P.S. W.V.L., X.S., and J.M.A. reviewed and revised the manuscript. All authors read and approved the final version of the manuscript.

Declaration of Competing Interest

None reported.

Ethics Approval and Consent to Participate

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based MEtabolome-TRanscriptome Correlation Analysis algorithm and uncovered LGG- and trp-dependent metabolites that were positively or negatively correlated with fatty acid metabolism and β-oxidation gene networks. This high-throughput metabolome-transcriptome correlation strategy can be used in similar investigations to reveal potential interactions between specific metabolites and functional or disease-related transcriptomic networks.

Keywords

Lactobacillus rhamnosus ; lipid metabolism; microbiota; obesity; probiotic; tryptophan

Introduction

There is a growing body of studies aimed at understanding the impact of gut microbiota on metabolic and inflammatory diseases. Since 2000, more than 4000 studies and almost 200 clinical trials have associated obesity with certain gut microbiota profiles.[1] The relative abundance of bacterial species in the Bacteroidetes phylum, the Christensenellaceae family, and the genera of Methanobacteriales, Lactobacillus, Bifidobacteria, and Akkermansia decreases in obesity.[1,2] The relevance of gut microbiota composition to systemic obesity was clearly demonstrated when germ-free (GF) mice inoculated with microbiota from 4 sets of obese or lean human identical twins took on microbiota characteristics of the donor: those mice receiving microbiota from the obese twin had an increase in adiposity, whereas those receiving “lean” microbiota remained lean, even though both groups of mice ate similar amounts of the same diet.[3] Moreover, supplementation with Christensenellaceae bacteria reduced adiposity gains in GF mice inoculated with feces from obese humans.[4] Gut microbiota affects systemic obesity by influencing the efficiency of calorie harvest from the diet, and the process of energy usage as well as storage.[5] The widely consumed probiotic *Lacticaseibacillus rhamnosus* GG (LGG) protects mice from obesity-associated, fructose-induced fatty liver via an increase in beneficial bacteria, restoration of gut barrier function, and subsequent attenuation of liver inflammation and steatosis.[6] Transcripts of inflammatory bowel disease-associated genes have also been linked to the abundance or predominance of certain pathobiont bacterial species in the gut microbiota of inflammatory bowel disease patients and animal models.[7–9]

The advent of metabolomics research over the past 10 years suggested that most effects of gut microbiota on human physiology may arise from metabolites that either directly emanate from microbes or are derived from host cells impacted by microbial colonization. Although certain microbial species or communities have been significantly associated with metabolic and inflammatory diseases leading to a “one-microbe-one-disease concept,”[10] distinctly different microbes or microbial groups can also have associations with similar disorders. This is likely due to shared metabolic pathways among gut microbiota; therefore, a marked change in gut microbial composition may only cause modest changes in their metabolic output and impact on host metabolomes.[11] Thus, there have been proposed associations between gut microbiota-derived metabolites and inflammatory disorders,[12] including obesity.[13] For example, human obesity is associated with leaky gut and low levels of microbiota-derived indole-3-propionic acid, a tryptophan (trp) derivative we

and others have observed to improve intestinal barrier and prevent diet-induced obesity (Suntornsaratoon et al, unpublished, 2023[14]). Several indole derivatives produced via trp transformation by the microbiota may have a role in the pathogenesis of metabolic syndrome, including obesity.[15]

Deconvoluting the complicated networks of microbe-associated metabolites and cellular physiology is challenging. First, a single commensal microbe may produce and alter the production of hundreds of metabolites in the host. Second, the abundances of host and microbial metabolites can be markedly influenced by diets and can vary not only among intestinal regions but also among various organ systems. Distinct dietary nutrients may alter the in vivo metabolomic profiles resulting from host-microbe interaction. For example, trp is an essential amino acid and is typically catabolized by the gut microbiota to indoles. A trp-deficient diet was shown to increase host susceptibility to intestinal and systemic inflammation, which could be mitigated by high-trp diets.[16–18] Intestinal dysbiosis is strongly associated with dietary trp deficiency, in part because host- or microbe-derived trp metabolites act as important signaling molecules to regulate host–microbial interactions and intestinal homeostasis.[19] Third, the complexity of metabolomic impact on host physiology can astronomically increase as the complexity of microbial community increases. Thus, a more sophisticated high-throughput experimental system is required to uncover and validate important links between microbial metabolites and host signaling pathways.

In this study, we took advantage of a reductionist approach to investigate if we can identify significant metabolomic and transcriptomic correlations in vivo by examining a single probiotic LGG,[20,21] with the alteration of a single nutrient component, dietary trp. Gnotobiotic mice that were fed with a trp-deficient or -containing diet were monoassociated with LGG or gavaged with phosphate buffered saline (PBS). After prolonged association, we simultaneously obtained the metabolomic and transcriptomic profiling data from individual mice. We then developed an R-script-based bioinformatic platform, named Metabolome-TRanscriptome correlation analysis (METRCA), which enabled us to conduct high-throughput analysis and identify significantly correlated host genes and metabolites. Using this experimental and bioinformatic approach, we discovered robust associations between LGG- and trp-associated metabolites with host gene networks belonging to the metabolic regulation of intestinal fatty acid processing and β-oxidation. These associations have not yet been previously reported—although LGG was found to reduce intestinal fat absorption, the proposed mechanism involved competitive consumption of dietary fatty acids, thereby reducing nutrient availability to host mucosal cells.[22] In addition to untargeted correlation analysis, this platform allows pathway-specific search for positively or negatively correlated LGG- or trp-dependent metabolites. We propose that this experimental approach will potentially help reveal unexpected regulatory links among any given microbe- or nutrient-associated metabolites to host metabolic or disease-related pathways.

Materials and Methods

Animals

C57BL/6 GF mice were purchased from Charles Rivers Laboratories (Wilmington). All mice were transferred to a sterile gnotobiotic individually ventilated caging system and

maintained in a controlled environment of 23 °C ± 1 °C, on a 12:12-hour light-dark cycle in a gnotobiotic facility at Rutgers New Jersey Medical School. After a 7-day acclimatization, mice were divided into 2 sets of 2 cages each (n = 5 mice). They were fed with either sterile trp-free (trp−) or trp-sufficient (trp+) diets (Research Diets) (Supplementary Table S1), with all other matching ingredients. After 2 weeks on designated diets, 1 cage from the trp-deficient (LGG−) and 1 cage from trp-sufficient (LGG+) were gavaged (200 μL) with LGG (ATCC 53103) at 10^8 CFU/mL per mouse, whereas the other cages were gavaged with 200 μL sterile PBS per mouse (PBS−, PBS+) (n = 5 for each group) (Fig. 1A). Three weeks after inoculation, all mice were sacrificed, then feces, serum, and ileum were collected and stored (−80 °C) for later analysis. Mice on trp-free diets were active, tended to consume more food, but lost a modest (<10%) amount of weight (Supplementary Fig. S1).

In a separate experiment, PBS- and LGG-gavaged trp-fed mice were compared with *Ruminococcus gnavus* (RG) monocolonized mice also sacrificed 3 weeks after inoculation and with conventional specific pathogen-free (SPF) mice, with both groups consuming trp+ diets (n = 4–5 for each group) (*R. gnavus* and SPF data obtained from[9,23]) (Fig. 1B). Animal procedures and protocols were conducted in accordance with the Rutgers University Institutional Animal Care and Use Committee.

To monitor gnotobiotic status, fecal pellets were collected once a week from each cage at pre- and at 1, 2, and 3 weeks post-inoculation, whereas colon contents were sampled at sacrifice, following earlier work.[24] Briefly, universal 16S rRNA quantitative PCR (Agilent AriaMx Real-time PCR system) was used for detection of total bacterial load in samples, if any, compared with negative control (distilled water) (16S rRNA forward `5′-AGAGTTTGATCCTGGCTCAG-3′`, and reverse `5′-GACGGGCGGTGWGTRCA-3′`). LGG was also quantified using LGG-specific primers (forward `5′-CGCCCTTAACAGCAGTCTTC-3′`, and reverse `5′-GCCCTCCGTATGCT TAAACC-3′`). The real-time PCR (qPCR) reaction was performed using SYBR Green (Thermo Fisher) as follows: 95 °C for 5 minutes, followed by 45 cycles at 95 °C for 10 seconds, 65 °C for 15 seconds, and 72 °C for 15 seconds, and a final extension at 95 °C for 5 minutes, 65 °C for 1 minutes, and 98 °C for 30 seconds.

All mice were validated as GF by IDEXX Laboratories Inc. before inoculation. qPCR analysis of colonic bacterial DNA using 16S rRNA or LGG primer sets indicated that PBS mice remained GF at sacrifice, whereas all LGG-inoculated mice (trp− or trp+) had similar LGG colonization abundances, regardless of diet (Fig. 1C). qPCR and culture analyses of LGG and RG feces were collected weekly in all cages and confirmed these results by in-house qPCR analysis.

RNA Isolation and Sequencing

Bulk-RNA sequencing analysis was performed on ileal RNAs from all mice. We chose the ileum because it has absorptive and metabolic functions qualitatively similar to the more proximate regions of the small intestine but in addition also has 2 unique properties: it is the site of bile acid resorption and of cobalamin transport. Moreover, bacterial load is greater in the ileum compared with proximal small intestinal regions. Thus, although we could have sequenced the other regions, we expected to discover more LGG effects here in the ileum. Illumina-compatible RNA-seq libraries were constructed using the NEBNext Ultra II

RNA library prep with sample purification beads (Cat. No. E7775) and NEB Next Multiplex Oligos for Illumina (dual index primers set 1; Cat. No. E7600) following manufacturer's instructions. Poly(A) selection and library quality were assessed using TapeStation 2200 (Agilent Technologies), and libraries were quantified using Qubit 4.0 fluorometer (Thermo Fisher). The prepared libraries were sequenced on an Illumina NextSeq 500 instrument (Illumina). CLC Genomics Workbench version 20.0.4 (Qiagen) was used for RNA-seq analysis, including identifying differentially expressed genes.

Extraction of Polar Metabolites From Feces and Serum

Frozen fecal matter was extracted (acetonitrile:methanol:water [40:40:20, v:v:v]) in 0.1 M formic acid (75 mL buffer per mg of sample). Samples were then sonicated (4 °C, 10 seconds) and centrifuged at 17,000*g* for 2 minutes. After centrifugation, the supernatant was diluted 4-fold with extraction buffer and then neutralized with 15% (m/v) NH_4HCO_3 before liquid chromatography–mass spectrometry (LCMS) analysis.

As for serum, polar metabolites were initially extracted by incubating samples with methanol (1:4 ratio) at −20 °C for 20 minutes. After centrifugation (17,000*g*, 2 minutes), the supernatant was transferred to new tube as first extraction, whereas the pellet was retained in the tube and later being extracted with extraction buffer for 10 minutes on ice (acetonitrile:methanol:water [40:40:20, v:v:v]). Thereafter, this solution mixture was centrifuged (17,000*g*, 10 minutes), and the supernatant was then combined with the first extraction. Extracts were stored at −80 °C for later analysis by LCMS.

Liquid Chromatography–Mass Spectrometry Metabolomics Analysis and Metabolites Identification

The LCMS analysis was previously described.[24] Briefly, analysis was performed on hydrophilic interaction chromatography coupled with electrospray ionization to the Q Exactive PLUS hybrid quadrupole-orbitrap mass spectrometer (Thermo Scientific), as previously described.[24] Thereafter, the targeted metabolite data analysis was performed in MAVEN.[25] The compound identification was based on the accurate mass and the retention time learned from in-house chemical collection. The untargeted analysis was performed in Compound Discoverer (Thermo Scientific). The metabolomics data sets were analyzed by MetaboAnalyst 5.0.

MEtabolome-TRanscriptome Correlation Analysis Correlation Analysis

The correlation analysis between metabolome (both negative and positive ionization mode of fecal and serum from 3 weeks after inoculation) and ileum transcriptome was performed using the R program (METRCA). Transcriptome data were filtered, and only the differentially expressed genes, as analyzed via CLC Genomics, with fold changes >1.5 and false discovery rate (FDR)-adjusted *P* values of <.05 were considered. In every comparison, pairwise Pearson correlation coefficients were calculated between genes and metabolites, and statistical significance of the correlation was obtained with the cor.test function in R. The *P* values were adjusted for multiple comparisons using the FDR method. Metabolite-gene pairs with FDR-adjusted *P* values of <.05 were considered to be significantly associated.

Statistical Analysis

Results are expressed as mean ± SEM. Statistical analysis of differences between the groups and the interaction of LGG and trp effect were analyzed by 2-way analysis of variance and Tukey's post hoc test. Statistical significance was *P* value of <.05.

Results

Lacticaseibacillus rhamnosus GG and Intestinal Fatty Acid Metabolism

Leading-edge plots from Gene Set Enrichment Analysis revealed that ileal genes involved in fatty acid metabolism (FAM) and oxidation (FAO) were among the top 10 pathways upregulated and significantly increased in LGG+ mice, with almost 70% of the entire FAM gene network elevated when compared with PBS−, PBS+, and LGG− mice (Fig. 1D, E). Upregulation of FAM genes required both LGG and trp and was not observed in other treatment groups. Other pathways significantly impacted by LGG-trp synergism mainly involved enterocyte function (brush-border transporters and enzymes, tight junction, apoptosis, protein secretion, and bile acid metabolism). This transcriptomic effect on host FAM was specific to LGG+ mice, as such an effect was not observed in mice monocolonized with *R. gnavus* and fed the same trp-containing diet. Approximately 55% of FAM genes remained elevated in LGG-associated mice, when compared with either *R. gnavus*-associated or conventionally raised, SPF mice (Fig. 1F). Many of the upregulated genes mediate important rate-limiting reactions in the FAM pathway (Fig. 2A).[26] For example, *Agpat1* (1-acyl-snglycerol-3-phosphate acyltransferase 1) and *Agpat2* are typically found in the endoplasmic reticulum and convert lysophaphatidic acid to phosphatidic acid and are thereby involved in biosynthesis of triglycerides and membrane phospholipids. *ApoB* codes for the primary apolipoprotein wrapped around the surface of chylomicrons, which are lipoprotein particles essential for exporting dietary lipids from enterocytes to the lymph and eventually systemic, circulation. *Dgat1* catalyzes the conversion of diacylglycerol and fatty acyl-CoA to triacylglycerol, whereas *Mgat2* not only catalyzes the formation of diacylglycerol (a triglyceride and phospholipid precursor) from 2-monoacylglycerol and fatty acyl-CoA but also mediates the rate-limiting step in intestinal triglyceride absorption.[27] In fact, intestine-specific deletion of *Mgat2* renders mice resistant to dietary fat-induced obesity.[27] *Gdp1* whose expression is enhanced in LGG-trp, regulates lipid metabolism by catalyzing the reversible conversion of dihydroxyacetone phosphate and NADH to glycerol-3-phosphate and NAD+. Therefore, *Gdp1* connects carbohydrate and lipid metabolism and participates in a glycerol phosphate shuttle that facilitates the transfer of reducing equivalents from the cytosol to mitochondria. *Slc22a5* is a carnitine transporter from the lumen into the cytoplasm, where carnitine is utilized in the transport of fatty acids from the cytosol into the mitochondria. Of these genes, *Agpat2, Dgat1, Dgat2, Gpd1*, and *Slc22a5* expressions increased specifically only in LGG mice fed trp, whereas *Agpat1, ApoB*, and *Mgat2* expressions increased in both LGG and SPF, but not in RG, mice (Fig. 2B). Thus, LGG in the presence of trp clearly appeared to uniquely promote the small intestinal abundances of these important fatty acid handling genes, a finding that has not been described previously.

Lacticaseibacillus rhamnosus GG and Fatty Acid β-Oxidation

We then analyzed the genes involved in FAO, which were among the top 2 gene sets upregulated in LGG+ mice (Fig. 1D). Like that observed for FAM genes, Gene Set Enrichment Analysis revealed a highly significant positive effect of LGG+ in promoting FAO genes (Fig. 3A). More than 60% of genes in this set were increased in LGG+ mice compared with that in PBS−, PBS+, or LGG− mice. These elevated FAO genes were also unique to LGG, as ~60% of them remained increased when compared with *R. gnavus*-associated or SPF mice (Fig. 3B). Representative enzymes that mediate important FAO reactions were increased only in LGG+ mice (Fig. 4A). With the exception of *Cpt1a* and *Cpt2*, such increases were unique to LGG-associated mice (Fig. 4B), including *Acaa2* that mediates the last step of mitochondrial FAO and breaks down fatty acids into acetyl-coA. *Acads, Acadm, Acadl* (not shown), and *Acadvl* initiate the degradation of short, medium, long-, and very long-chain fatty acids, respectively. All of these genes were significantly increased by 2-fold to 3-fold in LGG+ mice. In addition to FAO enzyme genes, *Cpt1a* and *Cpt2*, which participate in the carnitine shuttle essential in transporting long-chain fatty acids from the cytosol to the mitochondrial matrix for β-oxidation, were also strongly elevated in LGG+ mice. Furthermore, the increased *Ech1* is a peroxisomal protein that regulates the auxiliary step of fatty acid β-oxidation, whereas *Slc27a2* converts long-chain fatty acids into their acyl-coA esters, thereby initiating their degradation. LGG-enhanced upregulation was clearly dependent on dietary trp. In summary, LGG appeared to uniquely promote both FAM (Fig. 2B) and FAO gene networks (Fig. 4B).

Lacticaseibacillus rhamnosus GG Modulated Fecal and Serum Metabolomes

Principal component analysis revealed that LGG modulated the host fecal and serum metabolomes in the presence or absence of dietary trp. Dietary trp modestly separated the fecal total and fecal trp-related metabolomes of PBS-gavaged mice (compare blue and pink symbols, Fig. 5A, B). LGG monocolonization clearly resulted in the widening of the trp-induced separation. Serum trp-related metabolomes were primarily affected by dietary trp (Fig. 5B). In contrast, there was an overlap of the serum total metabolome between PBS and LGG mice fed trp-deficient diets that was separated by LGG monocolonization of trp-fed mice.

Metabolome-Transcriptome Correlations

The transcriptome results depicted above suggested that numerous FAM and FAO genes changed their expression as a function of LGG and dietary trp treatments (Figs. 1–4). As fecal and serum metabolites were also modulated by these treatments (Fig. 5A), we hypothesized that some of the transcriptomic and metabolomic changes were likely to be functionally correlated and that such correlations might be driven by unique metabolite-to-transcript or transcript-to-metabolite interactions. We hypothesized that genes within a metabolic or signaling pathway might be correlated with sets of metabolites. However, we were not able to find any platform that could perform high-throughput correlation analysis between our metabolomic and transcriptomic data sets that were derived from the same cohorts of animals. Therefore, we developed an R-script (METRCA) that performed and

identified significant metabolite–transcript correlation analysis by interrogating the ileum FAM or FAO transcriptomes to either fecal or serum metabolomes.

METRCA confirmed the importance of LGG-trp synergism in regulating FAM and FAO genes because only LGG+/PBS+ (effect of LGG in the presence of trp) and LGG+/LGG− (effect of trp in the presence of LGG) comparisons yielded metabolites that were linked with the greatest number of FAM or FAO genes (Fig. 5C, D). METRCA also revealed for the first time the important sidedness of metabolite regulation of enterocyte function, as fecal, compared with serum, metabolites were linked to a larger set of ileal FAM or FAO transcripts. It is important to note that METRCA could also be used to correlate the entire ileum transcriptome to the entire serum or fecal metabolomes to reveal untargeted correlations that would reveal just the total number of ileal genes each metabolite was associated with or the number of metabolites linked to each gene (Suntornsaratoon et al, unpublished, 2023).

We found that serum methylphenyllactate, methylnicotinamide, carnosine, 2-hydroxy-3-methylbutyric acid, leucic acid, uric acid, serotonin, kynurenine, L-arginino-succinate, 5-methoxytrp, and a few indoles were each associated with >60 FAM transcripts (Fig. 5C, S+ and S− columns). In contrast, more than 40 fecal metabolites were each correlated with >60 FAM transcripts (Fig. 5C, F+ and F− columns), suggesting that FAM modulation might occur primarily via the lumen. Interestingly, serotonin and indole-3-acetamide were the only metabolites linked to numerous FAM transcripts from both lumen (fecal) and serum, suggesting that either metabolite might regulate from the apical and basolateral membranes. Similar observations could be made for links between metabolites and FAO transcripts, indicating that FAM and FAO might be regulated by the same metabolites. Based on these METRCA results, we could then assess whether these metabolite–transcript correlations were positive (high levels of metabolite correlated with elevated transcript expression) or negative and could prioritize the experimental evaluation of each metabolite, depending on the number of genes it was linked with.

Effect of Lacticaseibacillus rhamnosus GG and tryptophan on Fatty Acid Metabolism, Fatty Acid Oxidation-Linked Fecal and Serum Metabolomes

A heatmap profile of the fecal metabolome revealed that LGG-trp synergism increased the levels of approximately 40% representative (obtained from Fig. 5C, D) metabolites (Fig. 6A). Far fewer serum metabolites were linked to FAM and FAO transcripts, and with the possible exception of adenosine, methylnicotinamide, carnosine, and methionine, their levels did not increase in LGG+ mice (Fig. 6B). The synergistic effect of LGG-trp on levels of fecal metabolites associated with FAM and FAO was depicted by 5-hydroxy-trp, carnitine, citrulline NG-dimethyl-arginine, salicylamide, and uric acid, whereas acetylmethionine and serotonin increased with dietary trp even in the absence of LGG (Fig. 6C). Almost all serum metabolites increased with dietary trp even without LGG, indicating that dietary effects predominated in this compartment (Fig. 6D). Among the few metabolites present in both compartments, fecal salicylamide increased with LGG-trp, but serum salicylamide was independent of treatment.

Evaluating MEtabolome-TRanscriptome Correlation Analysis by Associating Fatty Acid Metabolism and Fatty Acid Oxidation Genes With Representative Metabolites

Dgat1 and *Mgat2*, the 2 genes critical in regulating intestinal FAM, were significantly positively correlated with fecal metabolites 5-hydroxytrp and carnitine (Fig. 6E). Of note, trp deprivation alone (please compare PBS− blue with PBS+ pink circles, Fig. 6E) modestly reduced 5-hydroxytrp abundance (see also Fig. 5C) but had no impact on *Dgat1* and *Mgat2* gene expressions (see also Fig. 2A). The addition of trp in the presence of LGG (compare LGG− yellow with LGG+ orange squares) and the addition of LGG in the presence of trp (compare LGG+ with PBS+) resulted in striking increases of 5-hydroxy-trp and its gene targets *Dgat1* and *Mgat2*; hence, these simultaneous increases in metabolites and FAM genes were highly correlated. *Dgat1* and *Mgat2* transcripts were likewise associated with fecal carnitine. Both these fecal metabolites were significantly associated, in a similar manner, with the rate-limiting FAO transcripts *Acaa2* and *Cpt2* (Fig. 6F). These parallel correlations of fecal metabolites with both FAM and FAO genes suggest possible coregulation of these pathways by bacteria-associated fecal metabolites in vivo.

Although there were slightly higher levels of carnitine in the serum compared with those in the feces, serum carnitine levels did not differ significantly among treatments, and thus, no significant correlations could be found between this serum metabolite and FAM and FAO genes (Fig. 6G, H). Thus, significant interactions between an ileal FAM or FAO transcript and LGG-associated metabolites may be dependent on their tissue location.

A unique correlation is exemplified by serum serotonin (Fig. 6G, H). Although serum serotonin levels increased in both PBS+ and LGG+ groups (Fig. 6D), *Dgat1, Mgat2, Acaa2*, and *Cpt2* only increased significantly in LGG+ mice, suggesting that serotonin regulation of these genes, which occurred only when LGG was present, might require a LGG-associated cofactor.

Fatty Acid Metabolism and Fatty Acid Oxidation Gene Correlations With Representative Metabolites

Each metabolite was often simultaneously correlated with several genes as indicated above. Here, we identified that fecal metabolites indole-acetamide (IAM), carnitine, 5-hydroxytrp, and N-acetyl-glutamate were markedly significantly and positively linked to various representative FAM and FAO genes (Fig. 7A). In contrast, fecal 2-phenylglycine was negatively correlated with mostly FAM but fewer FAO genes. Thymidine, guanosine, and citrate were also negatively correlated (not shown). Fecal carnitine correlated with a larger number of FAM genes than with FAO genes. Other fecal metabolites strongly positively correlated with FAM genes were N-acetyl-glutamine, salicylamide, and nicotinamide riboside, whereas indole-carboxyaldehyde, indolelactic acid, serotonin, and trp displayed fewer, less robust positive correlations (not shown). For FAO, N-acetyl-glutamine, salicylamide, indole-carboxyaldehyde, and nicotinamide riboside were additional positive correlators, whereas citrate was negatively correlated. Indole originates exclusively from bacteria because mammalian cells do not have the metabolic capacity for the production of this compound.[28] It is interesting to note that although numerous indoles were detected in the fecal compartment (Suntornsaratoon et al, unpublished, 2023), only fecal IAM and

indole-carboxyaldehyde were identified by METRCA to link to a significant number of FAM and FAO genes. Fecal salicylamide was likely made by LGG, as numerous bacteria can synthesize this compound.[29]

In contrast to fecal indoles, serum indoles appeared to be more associated with FAM and FAO genes (Fig. 7B). For example, serum indole-3-propionic acid, indole acetonitrile, IAM, indole-carboxyaldehyde, indolelactic acid, and indoleacrylic acid were robustly associated with FAM genes. These same indoles, except indoleacrylic acid, were also positively associated with FAO genes. Other serum metabolites positively associated with several FAM genes were methylnicotinamide, carnosine, methionine, serotonin, and trp (not shown). Serum uric acid was negatively correlated with FAM and FAO genes. Serum argininosuccinate, uracil, leucic acid, acetyllysine, 2-hydroxy-3-methylbutyric acid, and phenyllactic acid were also negatively correlated with these genes (not shown). Although some metabolites were found in both fecal and serum samples, the majority of metabolites were associated with intestinal FAM and FAO genes from only 1 compartment.

We also used METRCA to enumerate the metabolites that were correlated with certain target genes (Fig. 7C). For example, we searched for both positive and negative metabolite correlators for the FAM genes *Agpat1, Dgat1, ApoB*, and *Mgat2*, which were correlated with a similar set of fecal metabolites and a similar set of serum metabolites (that were different from fecal metabolites). These results suggest potential regulation of the FAM gene pathway by the same metabolites. IAM, indole-carboxyaldehyde, and serotonin were the only correlated metabolites shared by both fecal and serum compartments. Although abundant in the serum, the neurotransmitter serotonin could also be released into the lumen from enteroendocine cells in response to signals from the gut microbiota.[30]

Next, we used METRCA to identify significant metabolite correlators with key FAO genes (Fig. 7D), such as *Cpt1a, Cpt2, Acadl*, and *Acaa2*. Unlike FAM, serum indoles were less frequently associated with various FAO genes, whereas indoleacrylic acid was not at all linked. There were also more negative correlators of FAO genes. For example, the 2 negative correlators, serum glycerol-3-phosphate and argininosuccinate, were associated either with no or few FAM genes but were with more FAO genes.

Discussion

Different from previous attempts to correlate metabolites and genes,[31,32] our approach took advantage of monoassociated GF mice with a dramatically reduced microbial complication. We were able to visualize and link the LGG and dietary trp impacts on intestinal transcriptome and metabolome as related to FAM and FAO. The high-throughput metabolomic–transcriptomic correlation strategy shown in this study to investigate the impact of a single bacterial species and a single nutrient component on host physiology may be used as a paradigm for similar investigations.

We unexpectedly uncovered a strong influence of LGG on host intestinal fatty acid handling and metabolizing gene networks. Importantly, such influence was heavily dependent on dietary trp and appeared unique to LGG. The intestine absorbs dietary lipids via diffusion

and transporters and then repackages them as chylomicrons for export and distribution to other organ systems, primarily adipocytes and skeletal muscle that strip the chylomicrons of fatty acids before liver uptake. An emerging theme in obesity research is the assessment of the microbiota's role in influencing the host gut's ability to absorb and harvest energy from dietary fats, thereby impacting systemic physiology. Although this characteristic remains controversial,[33] GF mice are resistant to dietary fat-induced obesity, suggesting that gut microbiota may affect not only energy balance but also whole-body obesity.[34,35] Gut microbiota in fish, mice, and humans enhance host intestinal nutrient, specifically fat, absorption.[35–37] These studies, and the findings that conditioned media from gut bacteria increases gut lipid absorption genes,[35] make associations between microbial metabolites and intestinal lipid metabolism genes physiologically important. Our finding suggests that LGG- and trp-dependent metabolites drive host lipid metabolism may be important for an effective intestinal handling of lipids. Indeed, LGG supplementation in mice protected against diet-induced obesity and fatty liver disease.[6,38] Intestines of conventional mice with *L. rhamnosus* strains were thought to consume dietary energy sources to sustain their own cellular activities and fuel cell renewal.[39] Because a preponderance of LGG-associated metabolites upregulate genes coding for fat metabolism and oxidation enzymes, it is possible that the protective effect of LGG against obesity is mediated by its metabolites.

Our results on the theoretical impact of LGG metabolites on FAM and FAO should be investigated experimentally but are supported by findings in previous studies. The most studied class of metabolites, indoles, have been shown phenomenologically to possess immense potency in preventing metabolic disorders, particularly obesity.[40] Other supplements, such as carnitine and carnosine, and their analogs have also been demonstrated to prevent obesity or mitigate comorbidities like dyslipidemia and steatohepatitis, mostly in animal models.[41,42] The mechanisms underlying these effects are not clearly known and may be related to the correlations that these supplements have with key enzymes identified by METRCA. We also discovered many other novel compounds whose impact on intestinal FAO and FAM, and ultimately on systemic obesity, has not yet been investigated. The analytical approach utilized in this study may thus also be used to extract valuable information from metabolite and RNA-seq data sets in similar investigations, generating novel hypotheses that may discover new interactions between microbe- or nutrient-associated metabolites and host metabolic or disease-related pathways.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Data Availability

We have deposited our script at zenodo (https://doi.org/10.5281/zenodo.10258756) so that this will be available for all users to perform similar correlation analysis. We have also provided example data files to reproduce our analysis.

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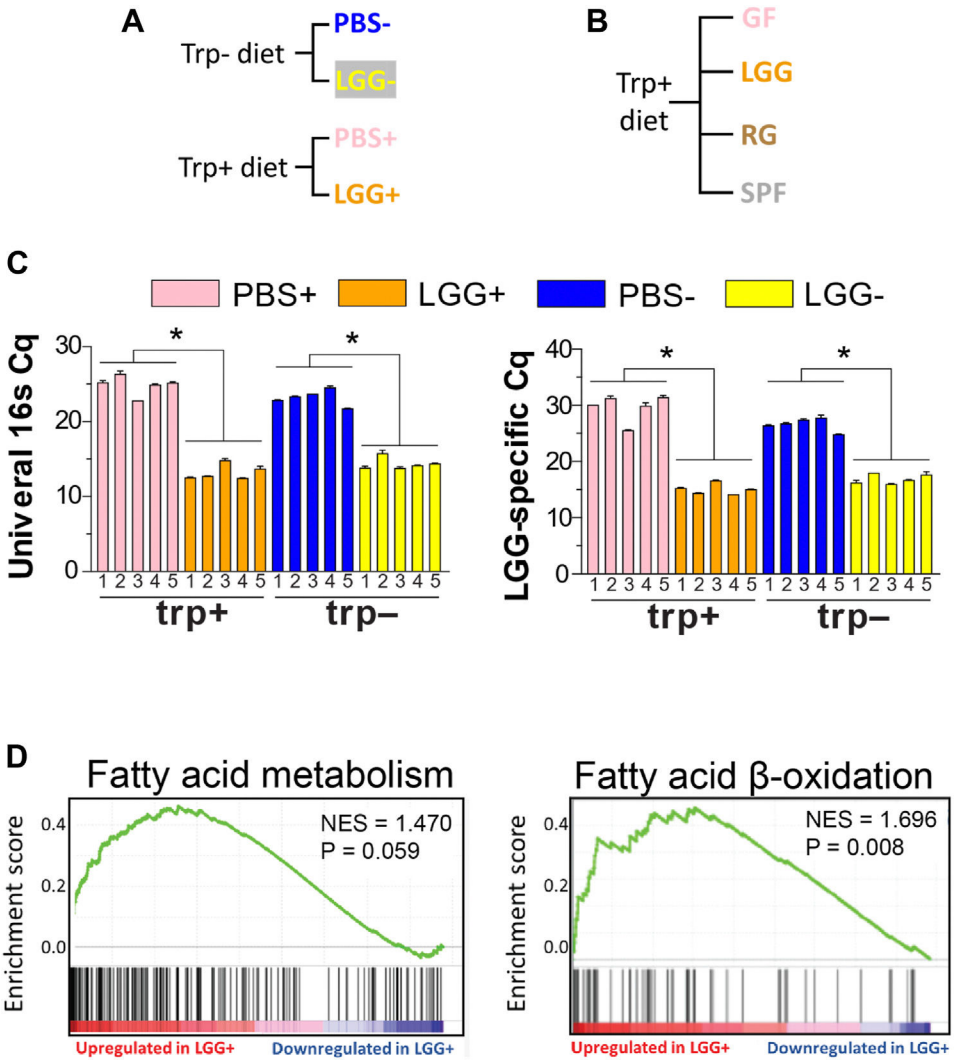


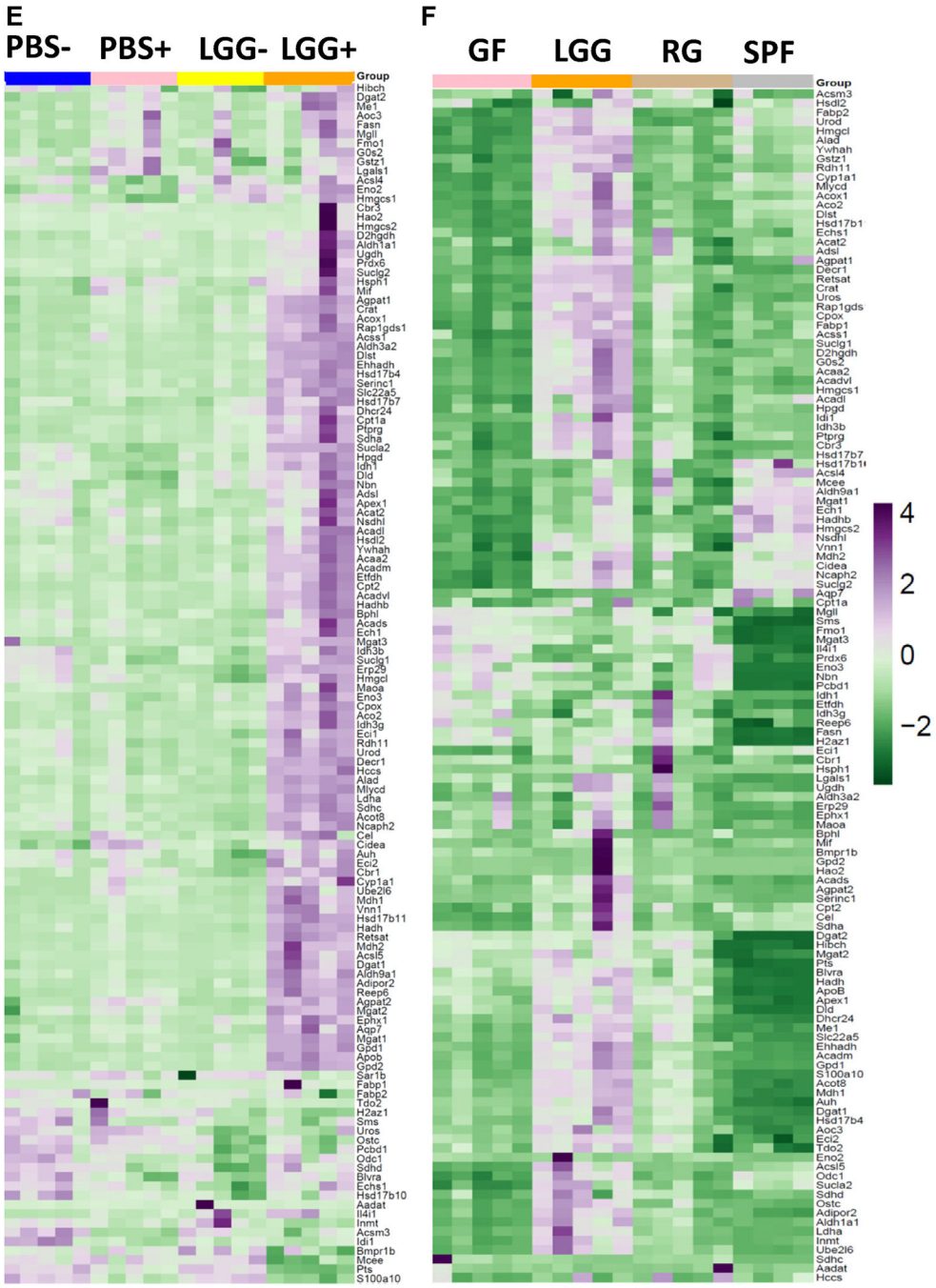


Figure 1.

The ileal transcriptome and fecal and serum metabolomes were collected from gnotobiotic mice fed tryptophan (trp)-containing and -free diets for 1 week and then monoinoculated with *Lacticaseibacillus rhamnosus* GG (LGG) (control PBS only) for 3 weeks, for a total of 4 treatments: PBS-gavaged fed trp-free (PBS−), LGG-gavaged fed trp-free (LGG−), PBS-gavaged fed trp (PBS+), and LGG-gavaged fed trp (LGG+) (A). Transcriptomes of LGG-inoculated (LGG) and PBS (germ-free [GF]) fed trp were then compared with those previously collected and analyzed in trp-fed mice monoinoculated with *Ruminococcus*

gnavus (RG) and mice raised in conventional cages (specific pathogen free [SPF]) (B). Determining GF status in mouse colon contents using universal 16S and LGG-specific primers. Colon contents were sampled at sacrifice. Water control had Cq's equivalent to PBS mice. Diet had no effect on LGG colonization. (C). Leading-edge graphs via Gene Set Enrichment (GSEA) indicated a robust enrichment of fatty acid metabolism (FAM) and oxidation (FAO) genes in LGG+ mice (D). Targeted heatmap analysis of transcripts of genes involved in FAM, obtained from bulk-RNA sequencing of PBS+, PBS−, LGG+, and LGG− ileal transcriptomes (E) and of trp-fed GF, LGG, RG, and SPF transcriptomes (F).

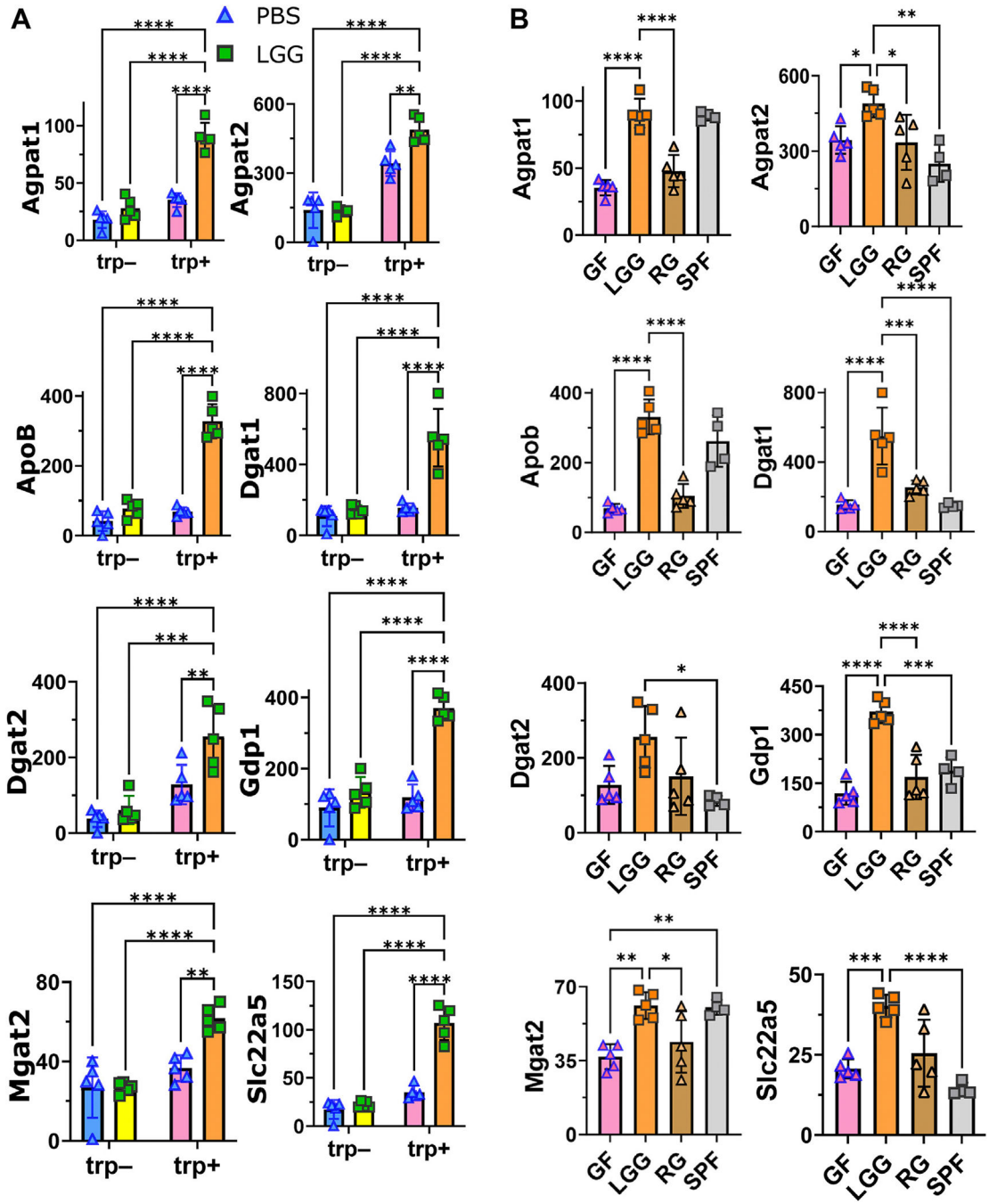


Figure 2.

Bar graphs of RNA abundances (in transcripts per million [TPM]) of representative genes involved in mediating important reactions in fatty acid metabolism (FAM). TPM is a better unit for RNA abundance because it respects the invariance property and is proportional to the average relative RNA molar concentration and thus adopted by the latest computational algorithms for transcript quantification.[26] The results indicated that many genes increased only in *Lacticaseibacillus rhamnosus* GG (LGG)-monocolonized and trp-fed mice, consistent with that exhibited in Figure 1E and F (A). For most FAM genes, this

effect was quite specific to LGG-tryptophan (trp) interactions, as their expression in trp-fed germ-free (GF), *Ruminococcus gnavus* (RG), and specific pathogen-free (SPF) mice was less (B).

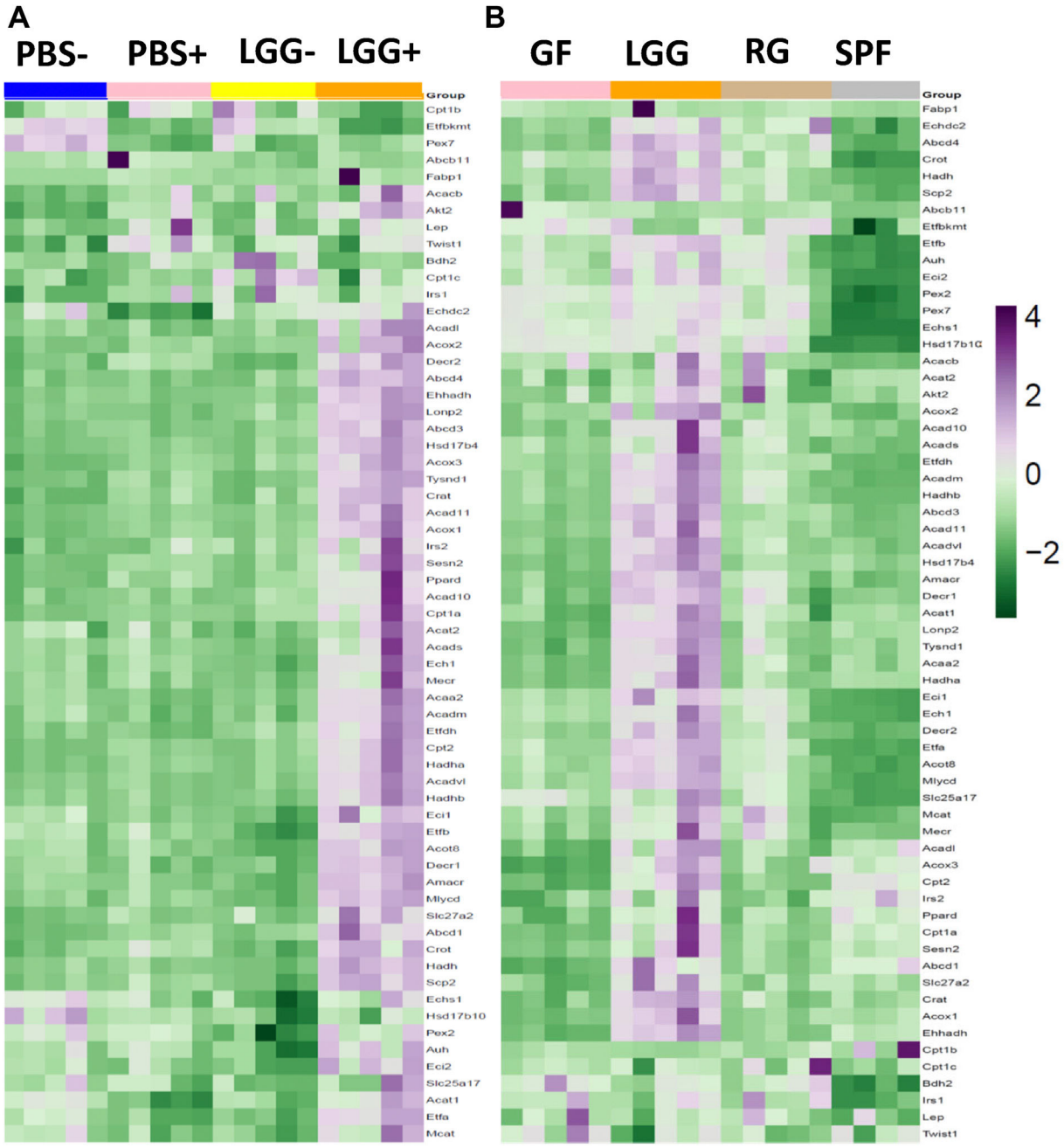


Figure 3.

Targeted heat map analysis of fatty acid oxidation (FAO) transcripts obtained from bulk-RNA sequencing of PBS+, PBS−, LGG+, and LGG− ileal transcriptomes (A) and of tryptophan (trp)-fed germ-free (GF), *Lacticaseibacillus rhamnosus* (LGG), *Ruminococcus gnavus* (RG), and specific pathogen free (SPF) transcriptomes (B).

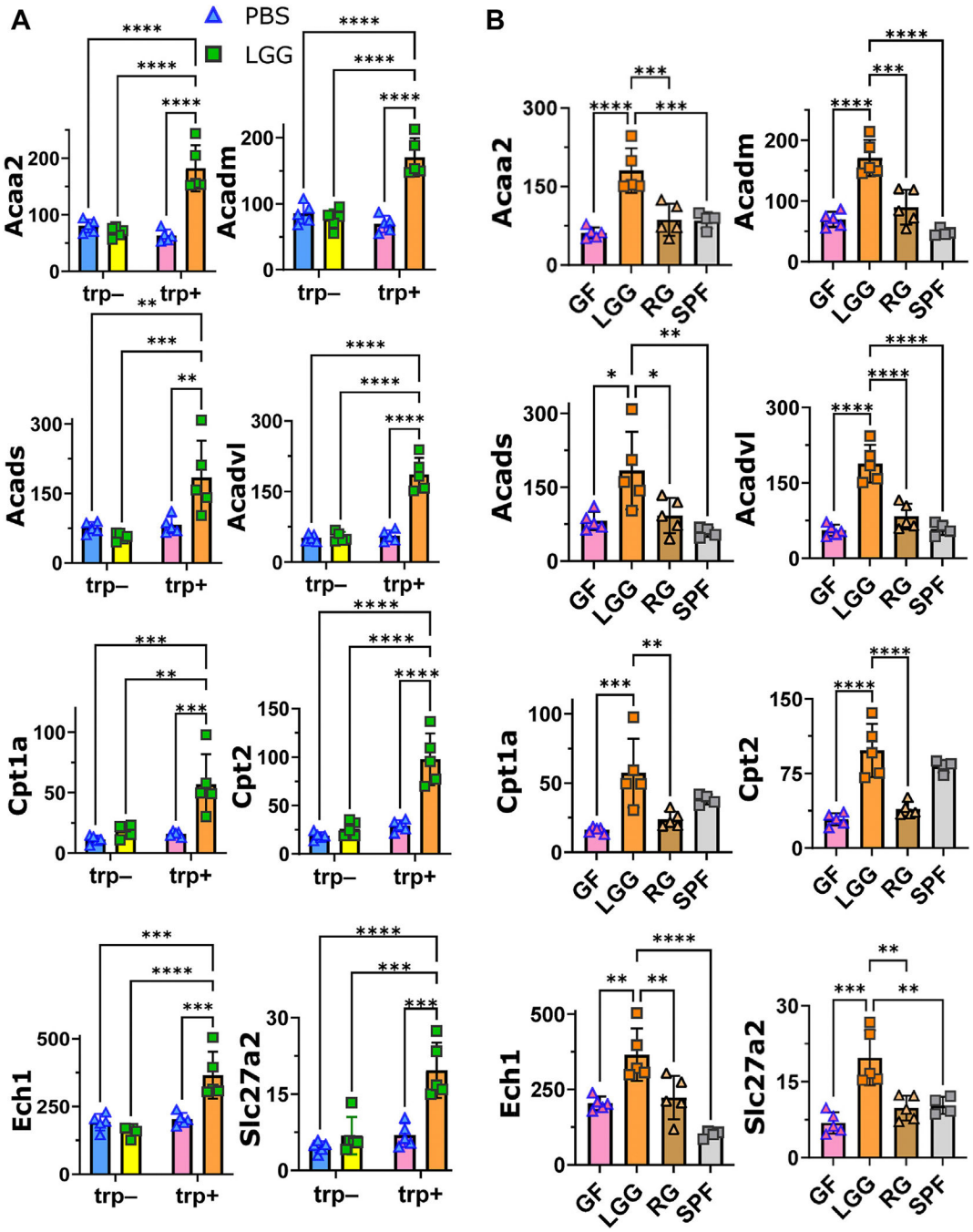
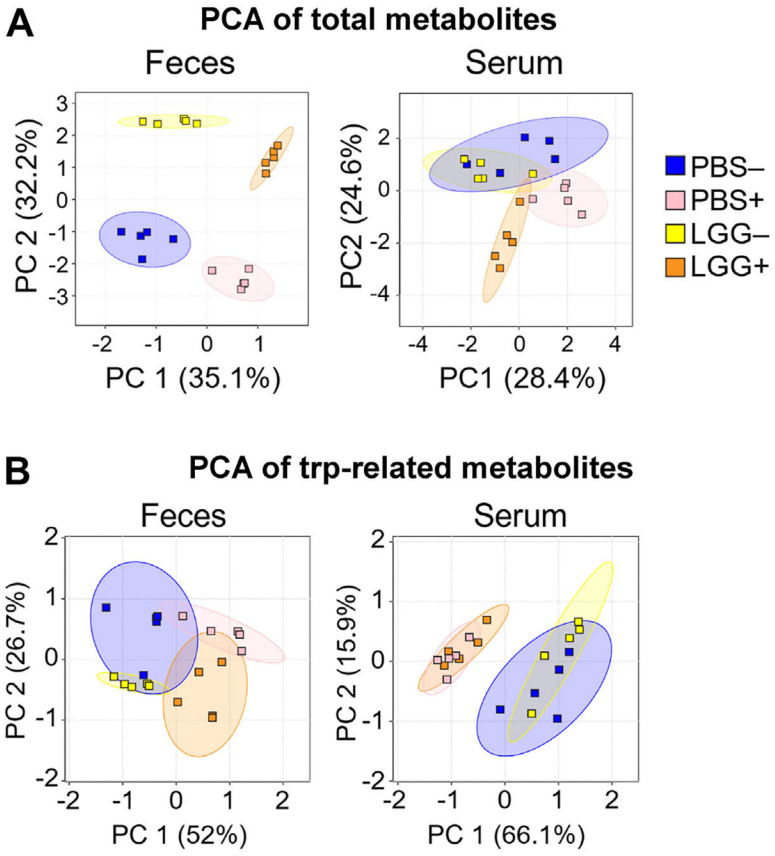
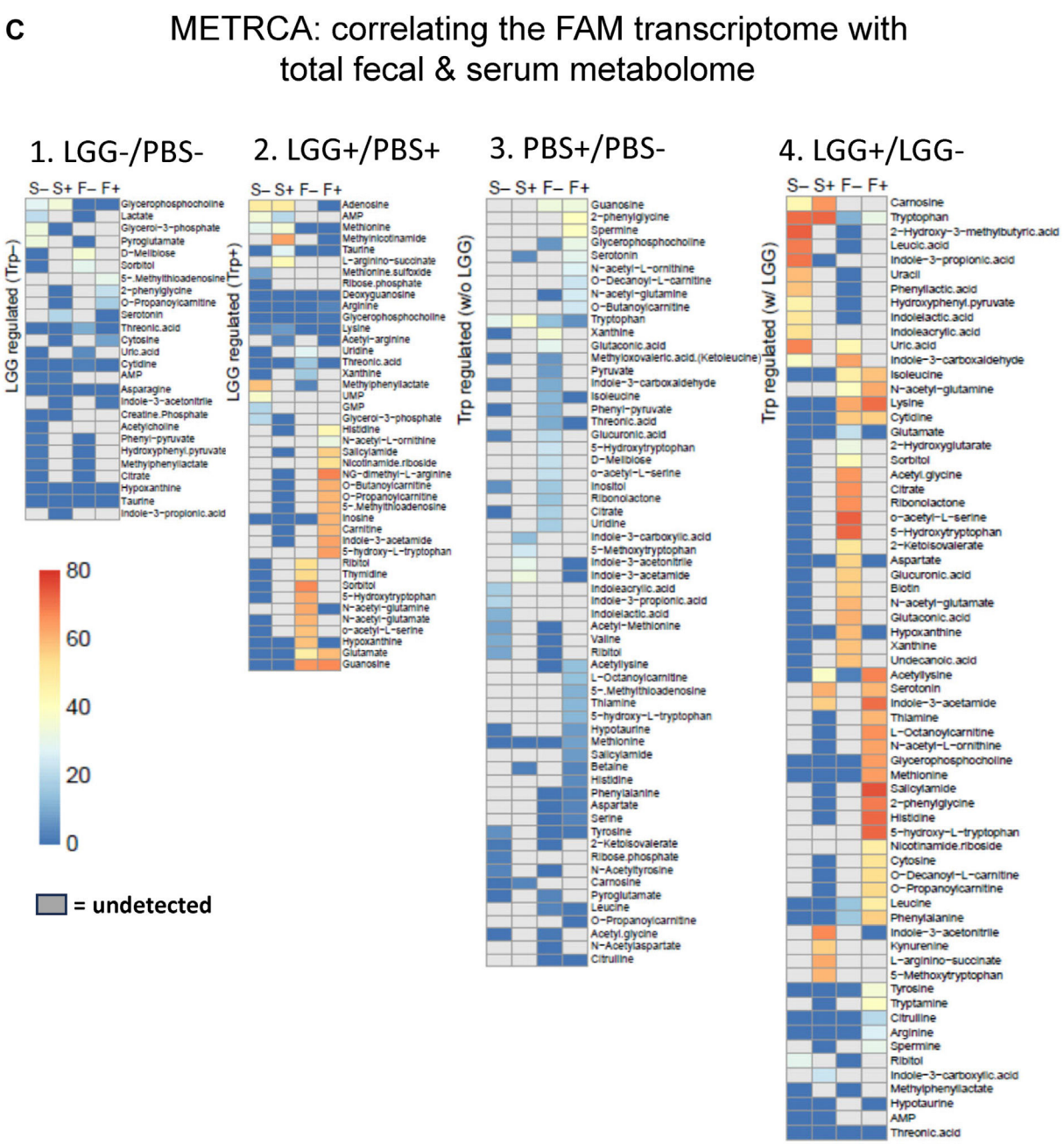


Figure 4.

(A) Bar graphs of representative genes involved in mediating important reactions in fatty acid oxidation (FAO) indicated that many genes increased only in *Lacticaseibacillus rhamnosus* (LGG)-monocolonized and tryptophan (trp)-fed mice, reflecting results shown in Figure 3. (B) This effect is quite specific to LGG-trp interactions, as expression in trp-fed germ-free (GF), *Ruminococcus gnavus* (RG), and specific pathogen-free (SPF) mice is less.





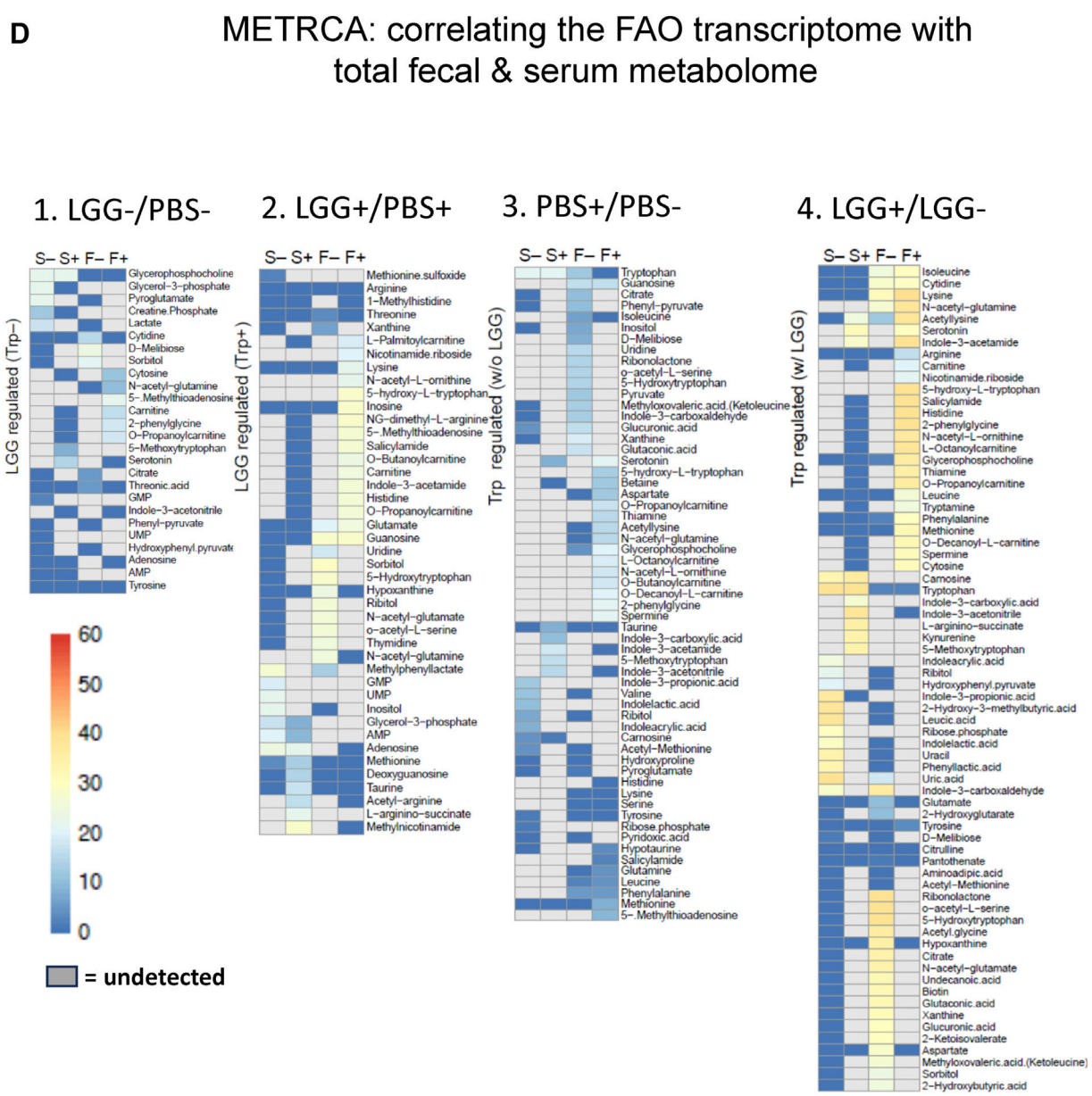
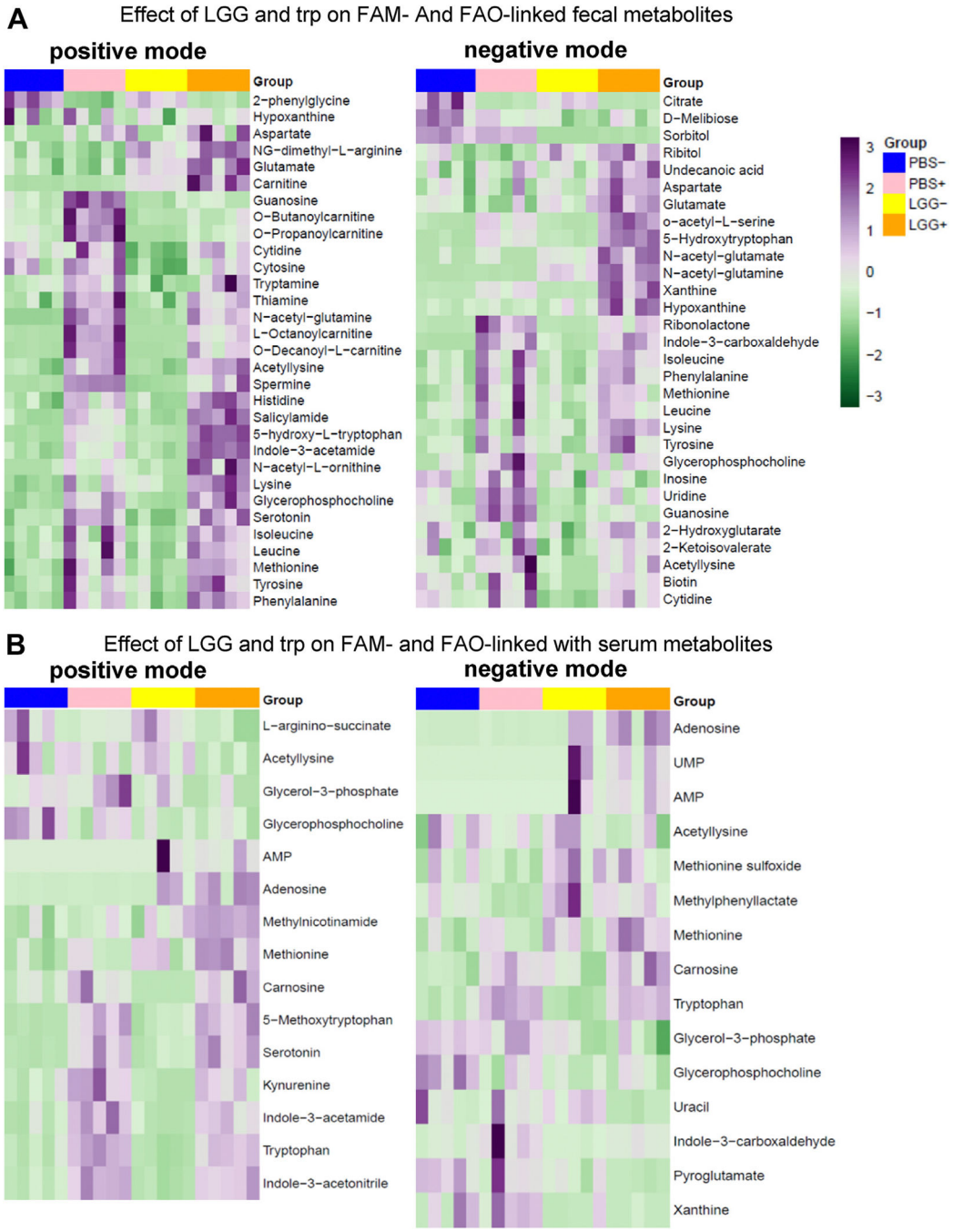
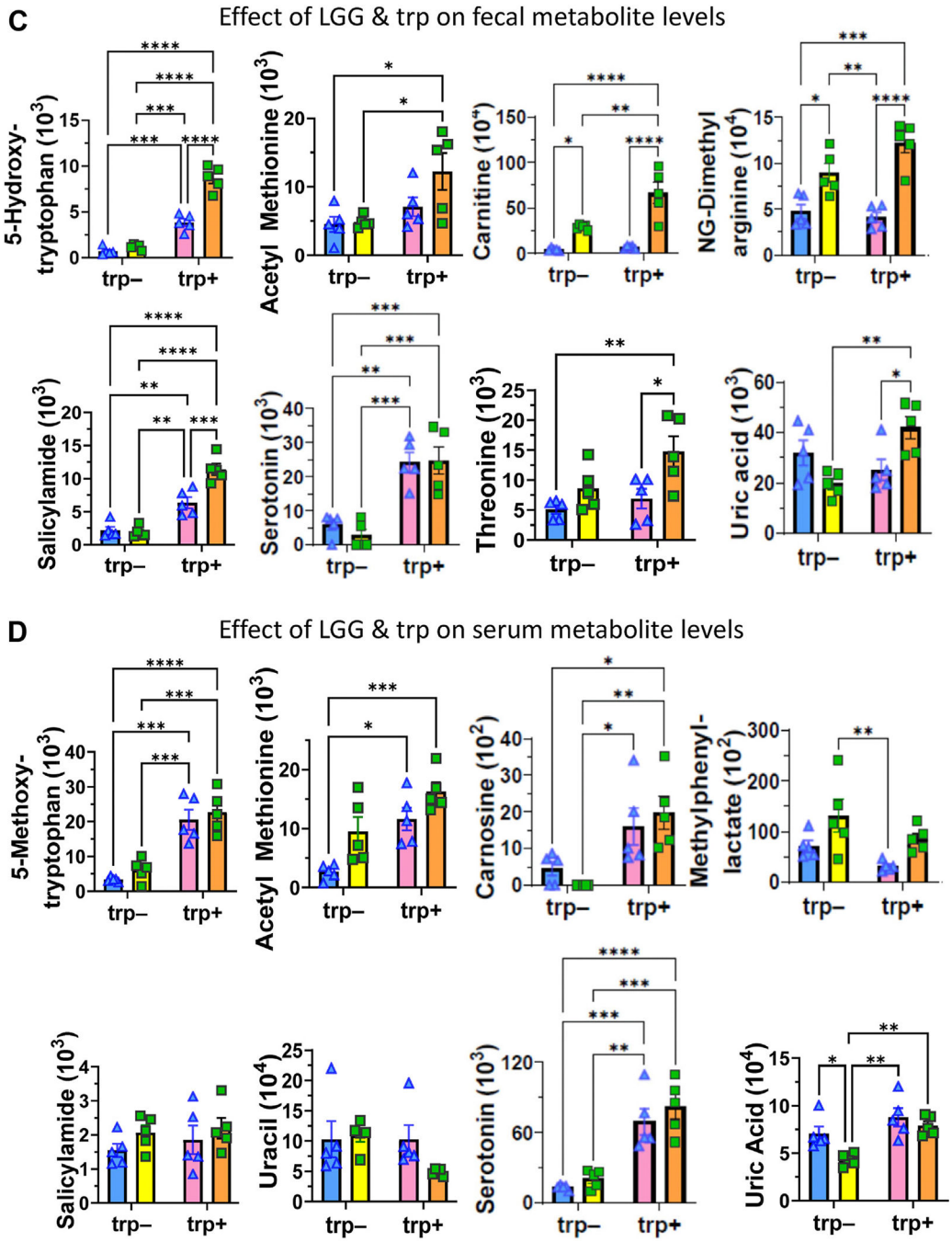


Figure 5.

Principal component analysis (PCA) of the total metabolome (liquid chromatography–mass spectrometry [LCMS]-positive mode) in the feces and serum of PBS−, PBS+, LGG−, and LGG+ mice (A). Similar results were depicted in negative mode metabolites (not shown). PCA of tryptophan (trp)-related metabolites (B). Because expression of many ileal transcripts varied with PBS−, PBS+, LGG−, and LGG+ treatments (eg, Figs. 2 and 4), whereas levels of numerous metabolites varied with those same treatments, we used MEtabolome-TRanscriptome Correlation Analysis (METRCA) to correlate the ileal fatty acid metabolism (FAM) and fatty acid oxidation (FAO) transcriptomes to the fecal and serum metabolomes. Heatmap depicting the numbers of FAM transcripts whose expression levels correlated with concentrations of specific metabolites (negative [−] and positive [+] modes) in serum (S) and feces (F) of LGG− compared with PBS− mice. These are

correlations regulated by *Lacticaseibacillus rhamnosus* (LGG) without trp (C1). Heatmap of correlations regulated by LGG with trp (LGG+ compared with PBS+) (C2). Heatmap of the number of significant metabolite–transcript correlations regulated by dietary trp in mice without LGG (PBS+ compared with PBS) (C3). Heatmap of the number of significant metabolite–transcript correlations regulated by trp in mice with LGG (LGG+ compared with LGG−) (C4). Heatmaps depicting the numbers of FAO transcripts whose expression levels changed as a function of LGG and trp (D). Note the large increase in number of FAM and FAO transcripts regulated when both LGG and trp are present. Dark blue rectangles mean no transcript can be correlated with that metabolite, and gray rectangles mean that metabolite was not detected by liquid chromatography–mass spectrometry (LCMS) in that condition.





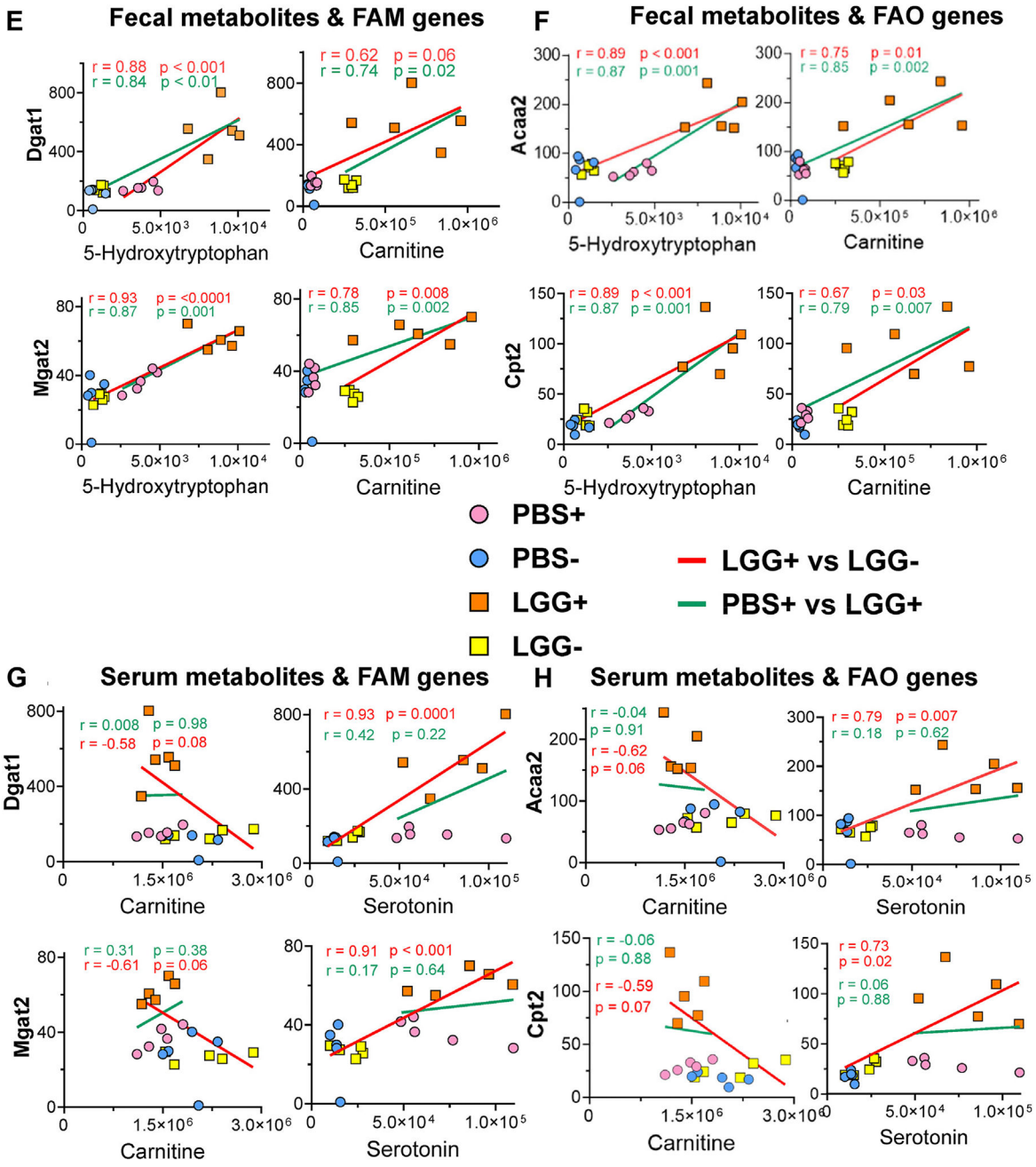
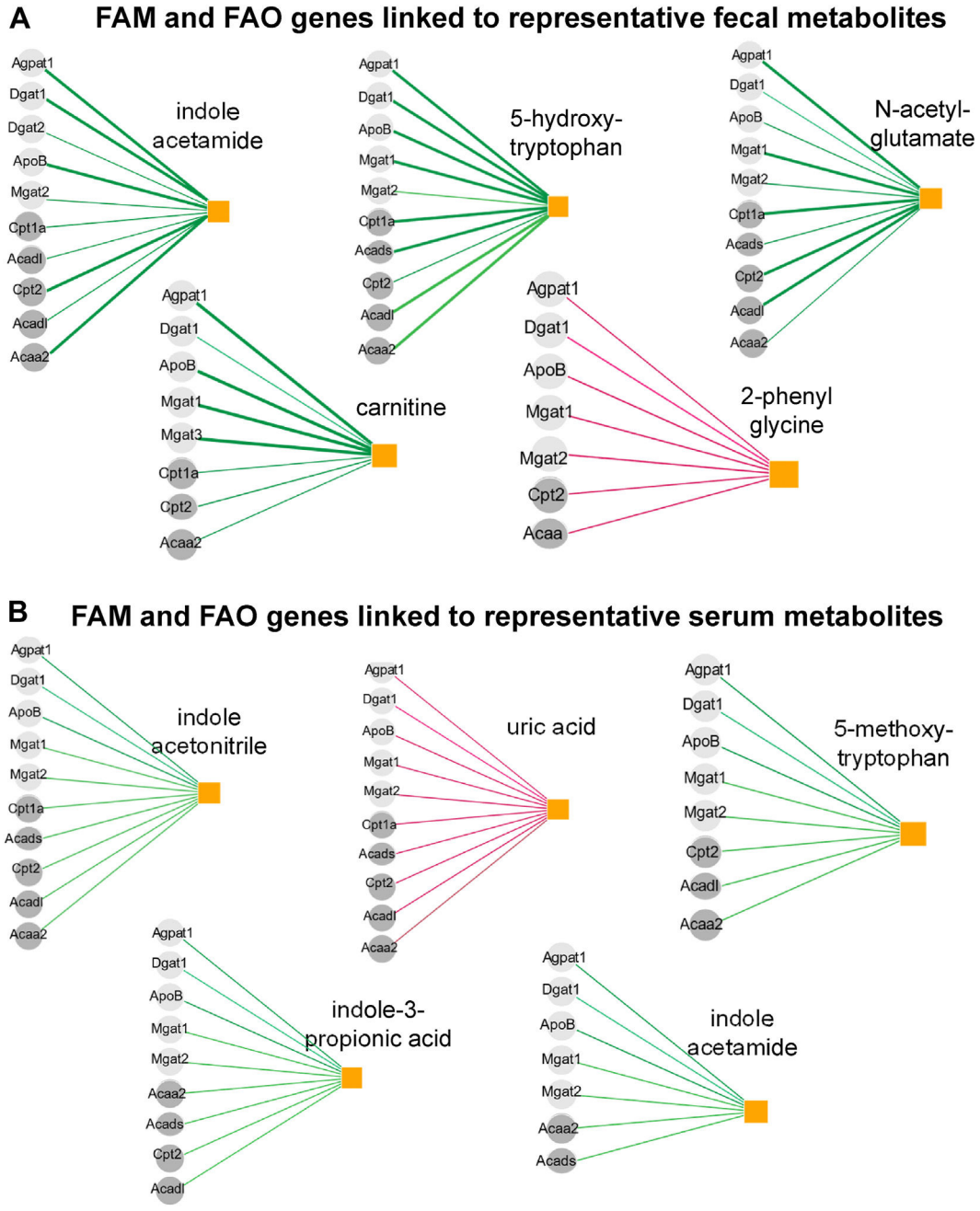


Figure 6.

Heatmap depicting the effects of *Lacticaseibacillus rhamnosus* (LGG) and tryptophan (trp) on relative levels of fatty acid metabolism (FAM)- and fatty acid oxidation (FAO)-associated metabolites (both liquid chromatography–mass spectrometry [LCMS]—and + modes) obtained from Figure 5C and D. A few of the fecal FAM-associated metabolites increased in PBS+ only, many increased in LGG+ only, and some increased in both treatments (A). Fewer metabolites were linked to FAO transcripts, and only adenosine, methylnicotinamide, methionine, and carnosine increased in LGG+ mice (B). Bar graphs of the effect of LGG and trp on representative FAM-associated (C) and FAO-associated

(D) metabolites. Representative Pearson correlations between selected fecal metabolites and FAM transcripts *Dgat1* and *Mgat2* (E), and with FAO transcripts *Acaa2* and *Cpt2* (F), and between serum metabolites and FAM (G) and FAO transcripts (H).



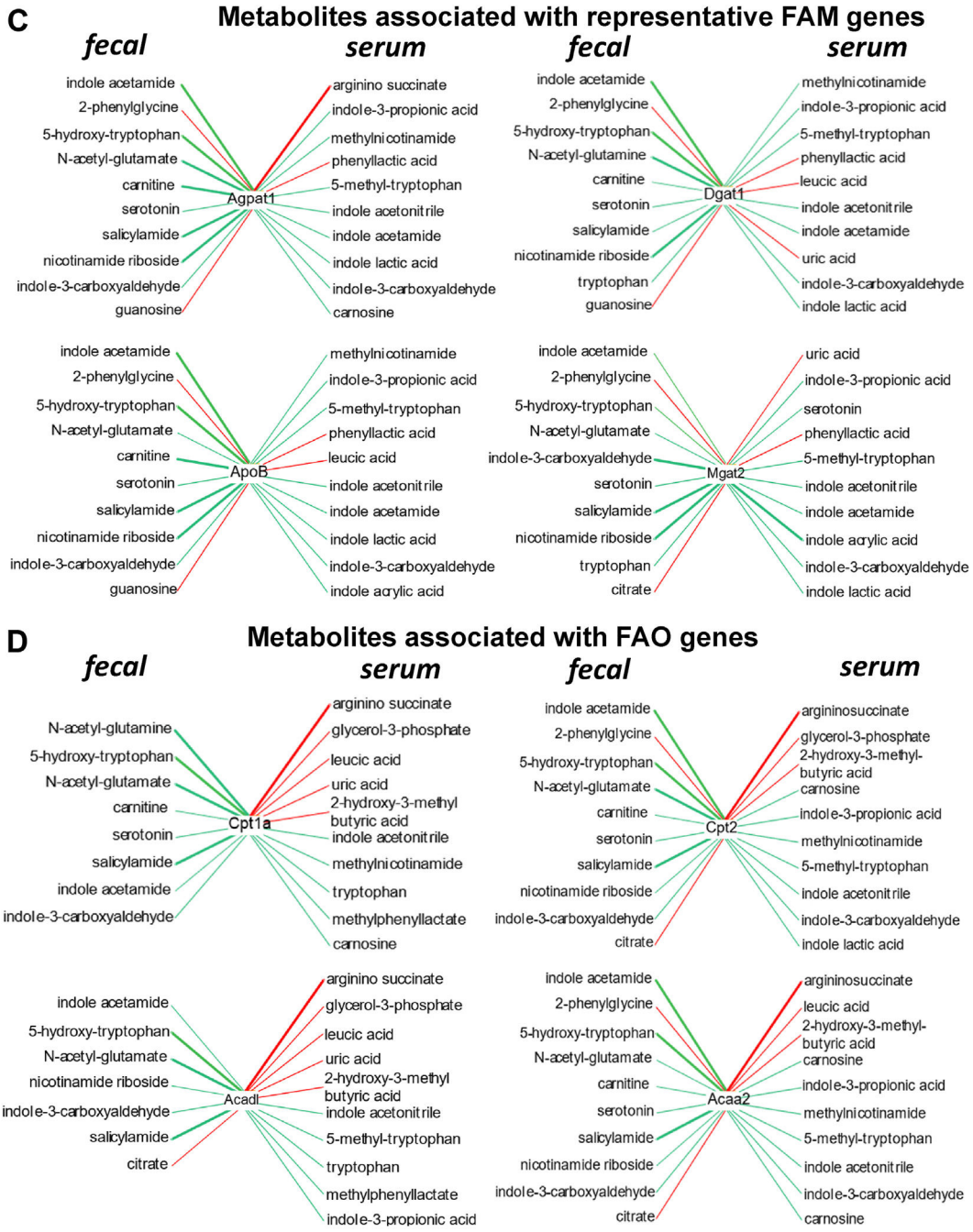


Figure 7.

In this diagram, we feature the links between 1 metabolite and up to 10 representative genes mediating pathways important in fatty acid metabolism (FAM) (light gray) and fatty acid oxidation (FAO) (dark gray). (The total number of genes correlated with each metabolite is in Fig. 5C, D.) Thick lines indicate significant MEtabolome-TRanscriptome Correlation Analysis (METRCA) correlations both between LGG+ and LGG− and between LGG+ and PBS+ mice; thin lines mean only 1 of the 2 correlations is significant. Green lines symbolize positive and red negative correlations. We examined correlations for the following

FAM genes: *Agpat1, Agpat2, Dgat1, Dgat2, ApoB, Mgat1, Mgat2*, and *Mgat3* and for the following FAO genes: *Cpt1a, Cpt2, Acaa2, Acads, Acadl*, and *Hsb17b10*. Fecal (A) and serum (B) metabolites correlated with representative FAM and FAO genes. Then, we feature significant associations determined by METRCA between a single gene and representative metabolites. Transcripts of important FAM (C) genes *Agpat1, Dgat1, ApoB*, and *Mgat2* that are significantly correlated by METRCA to fecal and serum metabolites and selected FAO (D) genes *Cpt1a, Cpt2, Acadl*, and *Acaa2*.