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Functional analysis of the relationship between intestinal microbiota and the expression of hepatic genes and pathways during the course of liver regeneration

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Abstract

Background & Aims—The pathways regulating liver regeneration have been extensively studied within the liver. However, the signaling contribution derived from the gut microbiota to liver regeneration is poorly understood.

Methods—Microbiota and expression of hepatic genes in regenerating livers obtained from mice 0 hour to 9 days post 2/3 partial hepatectomy (PHx) were temporally profiled to establish their interactive relationships.

Results—PHx led to rapid changes in gut microbiota that was reflected in increased abundance of Bacteroidetes *S24-7* and *Rikenellaceae* and decreased abundance of Firmicutes *Clostridiales*, *Lachnospiraceae*, and *Ruminococcaceae*. Phylogenetic Investigation of Communities by Reconstruction of Unobserved States (PICRUSt) was used to infer biological functional changes of the shifted microbiota. RNA-sequencing data revealed 6,125 genes with more than 2 folds difference in their expression levels during regeneration. By analyzing their expression pattern, six uniquely expressed patterns were observed. In addition, there were significant correlations between hepatic gene expression profiles and shifted bacterial populations during regeneration. Moreover, hepatic metabolism and immune function were closely associated with the abundance of *Ruminococcacea*, *Lachnospiraceae,* and *S24-7*. Bile acid (BA) profile was analyzed because bacterial enzymes produce BAs that significantly impact hepatocyte proliferation. The data revealed that specific bacteria were closely associated with the concentration of certain BAs and expression of hepatic genes.

Conclusions—The presented data established, for the first time, an intimate relationship between intestinal microbiota and the expression of hepatic genes in regenerating livers.

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Keywords

Bile acid; partial hepatectomy; metabolism; immune response; gut-liver axis

Introduction

Commensal bacteria are implicated in digestive tract health and disease. It is known that intestinal microbiota plays a role in regulating host cell proliferation and tissue repair [1, 2]. For example, germ-free mice have reduced intestinal epithelial cell turnover due to reduced proliferation, apoptosis, and crypt-to-tip cellular migration [3]. Germ-free mice also exhibit increased cancer incidence compared to conventional mice [4]. In addition, increased bacterial load and dysbiosis are found in colonic biopsies of patients with colorectal adenoma or cancer [5]. Moreover, gram negative bacteria-generated lipopolysaccharide (LPS) stimulates liver regeneration and tissue repair through toll-like receptor 4 (TLR4) signaling [6]. Gut microbiota also affects metabolic phenotype of the mammalian host and participates in microbial-host co-metabolism [7]. Alterations in gut bacterial communities are associated with metabolic disorders [8], metabolic syndrome [9], obesity [10-12], and nonalcoholic steatohepatitis [13]. There is an intrinsic link between proliferation and metabolism. Cell proliferation elevates metabolic demands to generate the energy and precursors for biosynthesis of macromolecules, and yet metabolic disorder dampens proliferation. Thus, intestinal microbiota, which is implicated in both proliferation and metabolism, may significantly regulate liver regeneration.

The liver is a major organ for host metabolism that can remarkably regenerate itself in response to partial resection or injury [14]. Liver regeneration requires activation of an array of genes and networks of signal transducers. Bile acids (BAs) have been identified as key metabolic signals during liver regeneration, and BA levels are tightly regulated by both host and microbiota [15]. There exists a “gut-liver axis” that facilitates bidirectional communication between intestinal microbes and BAs [1]. In one direction, the gut microbiota plays a pivotal role in regulating BA homeostasis. On the other end, BAs influence the gut microbiota profile. Although the bidirectional relationship of BAs and microbiota in the gut-liver axis has been investigated in humans and mice, whether it is linked to the regenerative process after liver resection remains largely unclear [16].

Previous studies have demonstrated the significance of BAs and its receptor farnesoid × receptor (FXR) in regulating liver regeneration [15]. However, the interplay between BAs, gut microbiota, and hepatic gene profiles during liver regeneration has not been defined. This is the first study to demonstrate the dynamic shift of hepatic transcripts and pathways in relation to gut microbiota as well as BA profiles in partial hepatectomy (PHx)-induced liver regeneration.

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Materials and methods

Animal experiments and sample collection

See Supplementary material and methods for sources of materials and methodological details.

Statistical Analysis

Data are given as mean ± SD. Statistical analysis was performed using Student's *t* test or one-way analysis of variance. Significance was defined by $p < 0.05$.

Results

PHx-induced liver regeneration

After 2/3 liver resection, liver mass was restored its original size at 7 to 9 days, consistent with previously reported findings (Fig. S1A) [17-19]. Ki67 immunostaining of liver sections revealed that cell proliferation started 1 day after PHx, peaked on day 2, and ceased on day 9 (Fig. S1B, C).

Alteration in microbial communities during liver regeneration

To characterize changes in the intestinal microbiota associated with regeneration, we constructed and sequenced16S rRNA amplicon libraries from cecal contents. Mice receiving PHx followed by wound closure and immediate killing (0 time point) were used as controls. Sham operation (Sham) followed by wound closure and immediate killing was also performed. Distinct changes in microbiota composition were noted during the course of regeneration (1 hour to 9 days) as compared to controls based on PCoA of taxon abundance data (Fig. 1A). The most abundant phyla consisted of Bacteroidetes and Firmicutes, which accounted for >95% of all sequences (Fig. 1B). Interestingly, Bacteroidetes abundance steadily increased while Firmicutes reciprocally decreased during liver regeneration (Fig. 1B). At lower taxonomic levels, *Clostridiales*, *Lachnospiraceae*, *Ruminococcaceae*, *Ruminococcus*, *Oscillospira,* and *Coprococcus* were the most abundant taxa within the Firmicutes phylum. Members of the families *S24-7* and *Rikenellaceae* were the most abundant representatives of Bacteroidetes phylum (Fig. 1C). Overall, Firmicutes contraction was linked to decreased *Clostridiales* (44.9% to 25.9, p=0.07), *Lachnospiraceae* (21.7% to 6.1%, p<0.001), and *Ruminococcaceae* (19.3% to 10.3%, p<0.01), while Bacteroidetes expansion was linked to *S24-7* (11.1% to 47.7 %, p<0.001) and *Rikenellaceae* (0% to 5.8%, p<0.001) enrichment during the course of liver regeneration (Fig. 1D). Gut microbiota of sham-operated mice was compared with that of controls, and there was no significant difference for the aforementioned five families between the two groups (Fig. S2).

To study the potential function of guy microbiota at each studied time, the Linear Discriminant Analysis (LDA) effect size (LEfSe) was applied to the relative abundance of Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways predicted by Phylogenetic Investigation of Communities by Reconstruction of Unobserved States (PICRUSt) [20]. In controls, the pathways with the highest three discriminative power were “Bacterial chemotaxis”, “Bacterial motility proteins”, and “Flagellar assembly” under Cell Motility

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category, followed by pathways under Membrane Transport category, including “ABC transporters”, “Secretion system”, and “Transporters”. Eight metabolic pathways were found in this group under Carbohydrate Metabolism, Enzyme Families, Lipid Metabolism, Metabolism of Cofactors and Vitamins, and Xenobiotics Biodegradation and Metabolism categories. At 1 hour, the pathway with the highest discriminative power was the “DNA repair and recombination proteins” under Replication and Repair category. In addition, “Mismatch repair” and “DNA replication proteins” as well as “DNA replication” were also noted. Under Cellular Processes and Signaling category, the “Cell cycle” and “Cell division” pathways also had significant discriminative power. Other biomarkers with significant discriminative power were “Lipopolysaccharide biosynthesis proteins” and “Lipopolysaccharide biosynthesis” pathways. Most strikingly, functional biomarkers in 1 hour post-surgery were mainly involved in the Metabolism pathways (61%, 41 out of 67 pathways) including “Energy metabolism”, “Nucleotide metabolism”, and “Carbohydrate metabolism”.

Day 2 data, when hepatocyte proliferation peaked, was also applied to LEfSe relative to controls. There were 24 and 64 pathways found in controls and Day 2 samples, respectively (Fig. 1F). In controls, the pathways with the highest two discriminative power were the “Transporters” and “ABC transporters” pathways under Membrane Transport category, followed by the “Bacterial motility proteins” and “Bacterial chemotaxis” pathways under Cell Motility category. Eleven metabolic pathways were found in controls, and they were Carbohydrate Metabolism, Metabolism of Cofactors and Vitamins, Xenobiotics Biodegradation and Metabolism, Metabolism of Terpenoids and Polyketides, and Lipid Metabolism categories. For Day 2 group, the highest discriminative power pathway was the “DNA repair and recombination proteins” under Replication and Repair category. In addition, “Homologous recombination”, “Chromosome”, “Mismatch repair”, “Nucleotide excision repair”, “DNA replication”, and “Base excision repair”, were also found in this group. Under Cellular Processes and Signaling category, the “Membrane and intracellular structural molecules”, “Pores ion channels”, “Cell cycle”, “Lysosome”, “Peroxisome”, and “Cell division” pathways were identified in this group. Again, functional biomarkers in Day 2 group were also mainly involved in Metabolism pathways (61%, 39 out of 64 pathways) (Fig. 1F).

We next studied functional differences in microbiota among all groups during liver regeneration. The biological function of microbiota derived from mice killed at zero time and Day 9 had the most significant differences compared with others (Table S1). Twenty-four pathways were distinct between zero time point mice *vs.* others. Most strikingly, microbiota derived from Day 2 mice were functionally unique in pathways that are associated with amino acid, xenobiotic, and biodegradation metabolism while microbiota from Day 3 mice had unique function in information processing specifically in interacting with G protein coupled receptor.

The gut microbiota modulates bile acid conversion [1]. The bacteria, which are responsible for BA deconjugation, oxidation, and 7-dehydroxylation, were analyzed at the genus level [1]. The abundance of *Ruminococcus*, *Bifidobacterium*, *Lactobacillus*, *Clostridium* peaked 1 hour and 7 days after PHx (Fig. S3).

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Gene profiling in regenerating livers

RNA-Seq was adapted to study differential hepatic gene expression due to its high sensitivity and accuracy. RNA-Seq was performed twice using biological duplicated liver specimens and consistent data generated from two independent experiments were included for analysis. An average of 175 million reads was generated per liver and more than 80% of these reads were mapped to the mouse genome using TopHat. The mRNA levels of 6,125 genes changed more than 2 folds in at least one studied time compared to controls (0 hour), were used for two-way hierarchical clustering. The results showed that certain studied time points clustered together at different level. Days 1 and 2, when the first wave of DNA synthesis occurs, clustered together. Other clusters were Day 0 and 1 hour, which was the priming phase, Days 7 and 9, which represent the termination phase; and Days 3 and 5, when the second wave of DNA synthesis occurs (Fig. 2A) [14].

Compared to controls, Day 7 mice had the largest number of genes with altered expression levels (2,748 up and 1,665 down) followed by Day 9 mice (2,308 up and 1,521 down) (Supplementary Table 2). The number of genes with altered expression was similar in Day 2 (3,022), 3 (2,812), and 5 (3,942) mice. Mice killed at 1 hour had fewer changes compare to other groups (up 188 up and 93 down). The major molecular pathways modulated during liver regeneration were identified using DAVID pathway analysis program. The list of differentially expressed genes and top 3 pathways (based on the number of genes, $p<0.05$) were shown in Table 2. In the priming phase (1 hour), expression of genes involved in metabolic processes and transcription was induced, while those for carboxylic acid, organic acid, and lipid biosynthetic process were reduced. One to five days after PHx, genes regulating cell cycle, macromolecular complex assembly, and wound healing were induced. Genes involved in immune response were down-regulated at 1-2 days, but up-regulated at 5, 7, and 9 days. In contrast to the immune function related genes, transcription pathways-associated genes were up-regulated at early time point (1 hour), but down-regulated at later time points (7 and 9 days) (Table S2).

Because biologically related gene groups may exhibit similar expression patterns, we analyzed the expression pattern of hepatic genes by STEM (Short Time-series Expression Miner) [21]. STEM generated 30 profiles with 6 being statistically significant (Fig. 2B). Based on the number of genes, the most significant was profile 13 that had 818 genes with expression peaking on Day 2 (Fig. 2B, C). The second most significant profile was 29, which had 691 genes with expression levels peaking on Day 1 followed by profile number 4 and 25, which contained 558 and 340 genes with expression levels continuously declining or increasing during the course of regeneration, respectively (Fig. 2B, C). Gene Ontology (GO) analysis was performed to generate the top 10 pathways for each of those six profiles (Table S3). Profile 13 contained many genes involved in regulating intracellular non-membrane-bound organelle, chromosome, cell cycle, and DNA metabolic processes. Profile 4 contained genes with a role in autophagic vacuole assembly and metabolism whose expression levels continuously decreased during regeneration; whereas profile 25 included genes associated with plasma membrane structure, cell-cell junction etc. and their expression levels continuously climbed during regeneration.

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Association between gut microbiota and hepatic gene expression

Spearman correlation was employed to evaluate potential association between intestinal microbiota and hepatic gene expression using the most abundant bacterial families and genes included in the six significant profiles. The correlations between operational taxonomic units and hepatic genes with altered expression levels are represented in heatmaps (Fig. 2D). With the exception of profile 27, *Ruminococcacea*, *Lachnospiraceae*, and *Clostridiales* were tightly clustered suggesting an association with similar biological processes during liver regeneration. Interestingly, the *Ruminococcacea* and *Lachnospiraceae* families displayed a very similar pattern of correlations with the genes present in all profiles and these correlative patterns were in contrast to the patterns observed for Bacteroidetes *S24-7* family.

Since the most striking biomarkers shown during regeneration were involved in metabolism and microbiota impact host metabolism as well as immune response, we next analyzed the associations between abundance of bacterial families and expression of hepatic genes regulating metabolic and immunologic pathways. From RNA-Seq, 905 differentially expressed genes in metabolic pathways were chosen to correlate with microbiota data. Heatmaps show that the genes significantly correlated with at least one bacterial family (0.5 r –0.5, *p* 0.05, Fig. 3). Genes correlating with microbiota composition were involved in “Oxidative phosphorylation”, “Mitochondrial dysfunction”, “TCA cycle” by pathway analysis. Top 10 immune pathways correlating with bacteria abundance are shown in Supplementary Table 4, which included *NF-kB* signaling and crosstalk between dendritic cells and natural killer cells. Genes involved in these pathways were also listed in Supplementary Table 4, which includes toll-like receptor 4 (*Tlr4*), *NF-kB*, Fibroblast growth factor receptor 1 and 4 (*Fgfr1* and *Fgfr4*), *Cd44*, *Cd86*.

JAk2/STAT3, Wnt, TNF , MAPK/ERK1/2 are important early signals controlling liver regeneration[14]. The differentially expressed hepatic genes involved in those signaling pathways during the regeneration program were significantly associated with specific microbiota. These findings suggest that the gut microbiota may regulate liver regeneration through those signaling pathways (Fig. S4).

The relationship between gut microbiota and bile acid homeostasis

Potential correlations between gut microbiota and genes involved in BA pathways were examined due to their close functional association. The expression of 18 genes regulating BA metabolism changed more than 2 fold in at least one of the studied time points during regeneration in comparison to controls (0 hour) (Fig. 4A). These genes include a key BA regulator (small heterodimer partner, *Nr0b2*/*Shp*), 5 enzymes (*Cyp7a1*, cholesterol 7 alpha-hydroxylase; neutral cholesterol ester hydrolase 1, *Nceh1*; adenylate cyclase 7, *Adcy7*; epoxide hydrolase 1, *Ephx1*; 3-hydroxy-3-methylglutaryl-CoA reductase, *Hmgcr*), and 12 BA transporters (ATP-binding cassette, sub-family G, member 5: *Abcg5*; *Abcg8*; potassium intermediate/small conductance calcium-activated channel, subfamily N, member 2, *Kcnn2*; solute carrier family 10, member 1: *Slc10a1*/*Ntcp*; *Slc27a2*; *Slc10a2*/*Asbt*; sodium taurocholate cotransport peptide, *Oatp2*; *Slc22a7*; aquaporin 4, *Aqp4*; *Slc1a1*; bile salt export pump, *Bsep*; multidrug resistance gene, *Mdr1*). *Rikenellaceae* correlated positively

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with the expression of *Ncoh1* and negatively with *Bsep, Aqp4* and *Slc27a1,* while *S24-7* correlated positively with *Mdr1* expression. *Lachnospiraceae* correlated negatively with *Adcy7* expression and *Ruminococcaceae* negatively with *Cyp7a1* and *Adcy7* expression. *Clostridiales* members showed a significant positive correlation with *Shp*, *Abcg8,* and *Asbt* and a negative correlation with *Ncoh1* and *Mdr1* expression (Fig. S3).

The UFLC-MRM-MS was employed to analyze BAs. Total hepatic BAs were elevated immediately after liver resection (1 hour) indicating BA overload. Chenodeoxycholic acid (CDCA), beta-muricholic acid (β-MCA) and deoxycholic acid (DCA) concentrations displayed the highest increase among unconjugated BAs. Among tauro-conjugated BAs, conjugated α-MCA, β-MCA, and CDCA concentrations were also elevated at 1 hour after PHx. In cecal contents, DCA was increased after PHx, but ursodeoxycholic acid and cholic acid (CA) were decreased.

The relationship between the most abundant operational taxonomic units operational taxonomic units and BA concentrations was investigated. Lithocholic acid positively correlated with the Firmicutes genus *Ruminococcus* (*Ruminococcaceae* family) (r=0.46, p=0.02) and negatively with the Bacteroidetes family *S24.7* (r=−0.38, p=0.05). Interestingly, tauro-conjugated BAs showed opposite patterns of correlations between Firmicutes and Bacteroidetes members. T-α-MCA negatively correlated with *S24-7* (r=−0.40, p=0.04) and positively with *Clostridiales* (r=0.43, p=0.02). TCDCA and TDCA showed a positive correlation with *S24-7* (r=0.46, p=0.01; r=0.43, p=0.02, respectively) and a negative correlation with *Lachnospiraceae* (r=−0.41, p=0.01; r=−0.38, p=0.05, respectively). *Ruminococcaceae* also negatively correlates with TDCA levels (r=−0.41, p=0.03), Overall, hepatic secondary BAs positively associate with Firmicutes members and negatively with Bacteroidetes members, while tauro-conjugated BAs showed positive correlations with Bacteroidetes and negative correlations with Firmicutes.

In contrast to the liver, fewer correlations were found in the cecum, where *Ruminococcus* (*Ruminococcacea* family) and *Rumminococcus* (*Lachnospiraceae* family) showed a negative association with CA and CDCA concentrations, respectively (r= −0.40, p-0.04; r= −0.47, p=0.01, respectively) (Fig. 4C).

Discussion

The presented data, for the first time, analyzed changes in intestinal commensal microbiota occurring in mice whose livers are undergoing regeneration. Functional analysis demonstrated specific and unique functions of gut microbiota at each stage of liver regeneration. Accordingly, hepatic gene profiling also revealed unique expression patterns that can be associated with specific biological pathways involved in the regenerative process. Moreover, based on these unique functions of microbiota and hepatic gene expression profiles, their relationship was established. Furthermore, we demonstrated the significant role of BAs and their relationship to microbiota as well as their potential interactive effect in controlling liver regeneration.

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Firmicutes abundance was linked to decreased *Clostridiales*, *Lachnospiraceae*, and *Ruminococcaceae*, while Bacteroidetes expansion was linked to increased *S24-7* and *Rikenellaceae*. Firmicutes and Bacteroidetes are the two most dominant bacterial phyla affecting host energy extraction efficiency and linked with excess adiposity in both mice and humans [12, 22]. An imbalanced Firmicutes/Bacteroidetes ratio has been associated with various disease processes. For instance, obese mice have higher Firmicutes and lower Bacteroidetes density compared to lean mice. The decreased representation of Firmicutes in nonalcoholic steatohepatitis patients resulted from decreased *Ruminococcaceae* and *Lachnospiraceae* [23]. High-fat diet decreased *Ruminococcaceae* and increased *Rikenellaceae* in mice [24], and a higher *Rikenellaceae* abundance was noted in *db/db* mice compared to lean mice [25]. The increased unidentified operational taxonomic units in order *Bacteroidales*, family *S24-7,* one butyrate-producing bacteria, have been observed following exercise and in lean mice compared to obese mice [22, 26].

The predicted biological functions of the observed microbial community in mice were significantly different during critical stages of liver regeneration, such as hepatocyte priming and proliferation, compared to controls. The microbiota in control mice showed biomarkers involved in cell motility, membrane transport, and metabolism in carbohydrate, lipid, cofactors and vitamins as well as xenobiotics, which indicated maintenance of hepatic characters under normal condition. Surprisingly, within one hour, discriminative characters of microbiota involved in replication and repair, cellular processes and signaling category preceded induction of most other hepatic genes. Germ-free and antibiotic-treated mice exhibit impaired liver regeneration [27]. The data generated from bacteria functional analysis revealed that bacterial metabolites (e.g. LPS; folic acid; pyrimidine; purine; amino acids; vitamin B6; Ubiquinone; Nicotinate; nicotinamide; Terpenoids; Polyketides; Glycosaminoglycan; Streptomycin; Riboflavin) and signals (e.g. Cell cycle; Cellular Processes and signaling; Lipopolysaccharide biosynthesis; Replication and repair; Bacterial secretion system; Energy metabolism; TCA cycle; amino acid metabolism) generated by microbiota may play a role in liver regeneration. The gut microbiota has been referred to as a metabolic “organ” due to its immense impact on host physiology, metabolism, and immunity [28]. Within hours of liver resection, mice develop significant hypoglycemia and transient steatosis after one day. The amount of liver resected is positively associated with the extent of hypoglycemia, accumulation of hepatic triglyceride, and hepatocellular proliferation, which indicates co-regulation of metabolic responses and proliferation [29]. Furthermore, metabolites related to amino acids metabolism, also appear in the serum and accumulate in the regenerating liver [30].

The bacterial sequencing data generated identified enriched biomarkers mostly involved in metabolism appearing within one hour post liver resection, suggesting an initial response from the intestinal microbiota to meet additional metabolic demands. On the other hand, supplementation of additional nutrients such as glucose and high fat diet dampens liver regeneration [31]. It is possible that these additional nutrients further strained metabolic processes and thereby interfere with proliferative signaling. Thus, a fine-tuned metabolism as modulated by the intestinal microbiota as well as the host is critical for proper execution

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of liver regeneration. Our findings provide evidence that intestinal microbes-mediated metabolism occur prior to the proliferative phase of regeneration.

In addition to metabolism, pathways involved in LPS were up-regulated one hour post liver resection. The administration of gut-derived LPS induces hepatic DNA synthesis with LPS stimulating the release of several hepatotrophic factors such as insulin [32]. Conversely, mouse hepatic DNA synthesis is impaired when the channel permitting gut-derived LPS transport to the liver is blocked [33]. LPS administration rescues both germ-free and LPS-resistant mice from delayed liver regeneration [34]. Furthermore, LPS regulates innate immune response, which is intimately associated with liver disease and hepatic regeneration [35]. LPS binding to TLR4 for *NF-kB* activation, which is essential for the priming phase of liver regeneration [36]. Ampicillin-impaired liver regeneration is associated with increased of CD1d-dependent natural killer T (NKT) cells. The deficiency of NKT cells or interruption of CD1d-NKT interaction promoted hepatocyte proliferation [27].

Our data demonstrated a positive correlation between members of the gram-negative *S24-7* family and immune responses including *NF-kB* and natural killer cell signaling. In addition, gram-negative bacteria from *S24-7* and *Rikenellaceae* families, which produce LPS, expanded immediately following PHx. However, over-activation of immune pathways may exert adverse effects and thus, requires tight regulation. The opposite correlation patterns with genes linked to immune response and metabolic pathways displayed by Firmicutes (*Ruminococcacea* and *Lachnospiraceae*) and Bacteroidetes (especially *S24.7*) suggest their counter-balancing roles in fine-tuning these processes to reach a homeostasis beneficial for regeneration.

The major end products of bacterial fermentation in the gut are short chain fatty acids (SCFAs) including butyrate, acetate and propionate [37]. Butyrate provides energy for enterocytes, and acetate as well as propionate can be used for hepatic gluconeogenesis and lipogenesis [38]. Apart from their nutritional value, SCFAs regulate immune responses and hepatic metabolism [39]. SFCAs pool is regulated by diet as well as gut microbiota composition [38]. For instance, *Roseburia*, a genus within *Lachnospiraceae* family, is one of the main butyrate producers while propionate is mainly generated by *Bacteroides* species [40]. In addition, different SCFAs exert varying effects on the host [41]. Butyrate has been shown to improve insulin sensitivity and increase energy expenditure in mice [42]. Administration of propionate, as a satiety-inducing agent, resulted in a significantly greater feeling of fullness and lower desire for human to eat [43]. Because Firmicutes and Bacteroidetes abundance fluctuated during liver regeneration, it is important to quantify the amount of SCFAs and other bacterial metabolites in order to understand their potential contributions to liver regeneration.

It is interesting that although the liver has completely restored its original mass by day 9, microbiota composition remained altered. Gut microbiota, perturbed by dietary changes, antibiotics, or diseases, undergoes consecutive changes in composition and function until a relatively stable climax community is established. Transient perturbation of the microbiota by low-dose antibiotic in early life shows long-term metabolic effects [44]. The effect of clindamycin on *Bacteroides* in the gut lasted 2 years after treatment is completed [45]. With

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1-week of antibiotics treatment, the composition of gut microbiota in patients with dyspepsia shifted, persisting for up to 4 years without additional antibiotic treatment [46]. Whether the shifted microbiota composition detected in regenerating livers will recover or form a stable state needs to be further studied.

The mutual influences between BAs and gut microbiota has been of growing interest. Germ-free as well as antibiotic-treated animals have compromised liver regeneration and altered BA profiles [15, 27, 34]. In the PHx model, our data revealed a transient BA load increase 1 hour after surgery and a rapid return to baseline level at day 1, which is consistent with previous findings [15, 47]. Whether this abrupt change in BA load triggered the initial change or sustained the long lasting change found in microbiota profile remains to be investigated. The significance of BA in liver regeneration has been extensively demonstrated. BA receptor FXR knockout mice exhibit delayed liver regeneration due to dysregulated BA synthesis [24]. Intestinal FXR facilitates liver regeneration via up-regulation of FGF15/FGF19. In addition, intestinal FXR knockout mice have impaired liver regeneration due to insufficient FGF15 [21]. Moreover, hepatocyte-specific FXR KO mice also have delayed liver regeneration from CYCLIN D inactivation and suppressed HGF-mediated signaling [48].

Our data showed that the expression of multiple genes involved in BA homeostasis is altered in regenerating livers. Such changes were associated with abundance of certain intestinal microbiota taxa. The gut microbiota, by regulating ileal *Fgf15* expression through FXR and hepatic *Cyp7a1* via small heterodimer partner (*Nr0b2*, *Shp*), can cometabolize BAs [49]. Interestingly, our data showed a positive correlation between *Shp* expression and *Clostridiales* abundance, and a negative correlation between *Cyp7a1* expression and *Ruminococcaceae* abundance. Members of *Clostridium* cluster XIVa, including *Ruminococcacea* and *Lachnospiraceae*, are among the dominant groups of gut microbiota capable of producing secondary BAs through 7α/β-dehydroxylation [50]. Consistently, the concentration of hepatic LCA showed a positive and a negative association with *Ruminococcus* and *S24.7*, respectively. DCA concentration also positively correlated with the abundance of *Ruminococcus* but did not reach a statistical significance. The abundance of *Ruminococcacea* and *Lachnospiraceae* in association with secondary BA concentrations also has been reported in other models [50]. Lastly, hydrophobic BAs have been shown to promote proliferation [51]. To firmly establish the relationship between intestinal microbiota, BA homeostasis and liver regeneration, it is essential to identify the specific BA-producing bacteria.

In summary, the presented data indicate an extensive role of the intestinal microbiota in regulating metabolism as well as cell proliferation (Fig. 5). It is possible that initial alterations in BA profile shift gut microbial abundance and diversity in manners beneficial for liver regeneration.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

LPS	lipopolysaccharide
TLR4	toll-like receptor 4
BA	bile acid
FXR	farnesoid × receptor
PHx	partial hepatectomy
PCoA	Principal Coordinates Analysis
PICRUSt	Phylogenetic Investigation of Communities by Reconstruction of Unobserved States
STEM	Short Time-series Expression Miner
GO	Gene Ontology
Fgfr1	fibroblast growth factor receptor 1
Shp	small heterodimer partner
Cyp7a1	cholesterol 7 alpha-hydroxylase
Nceh1	neutral cholesterol ester hydrolase 1
Adcy7	adenylate cyclase
Ephx1	epoxide hydrolase 1
Hmgcr	3-hydroxy-3-methylglutaryl-CoA reductase
Abcg5	ATP-binding cassette, sub-family G, member 5
Kcnn2	potassium intermediate/small conductance calcium-activated channel, subfamily N, member 2
Ntcp	solute carrier family 10, member 1
Oatp2	sodium taurocholate cotransport peptide
Aqp4	aquaporin 4
Bsep	bile salt export pump
Mdr1	multidrug resistance gene
CDCA	chenodeoxycholic acid
β-MCA	beta-muricholic acid
DCA	deoxycholic acid

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UDCA	ursodeoxycholic acid
CA	cholic acid
NKT	natural killer T cell
SCFA	short chain fatty acid

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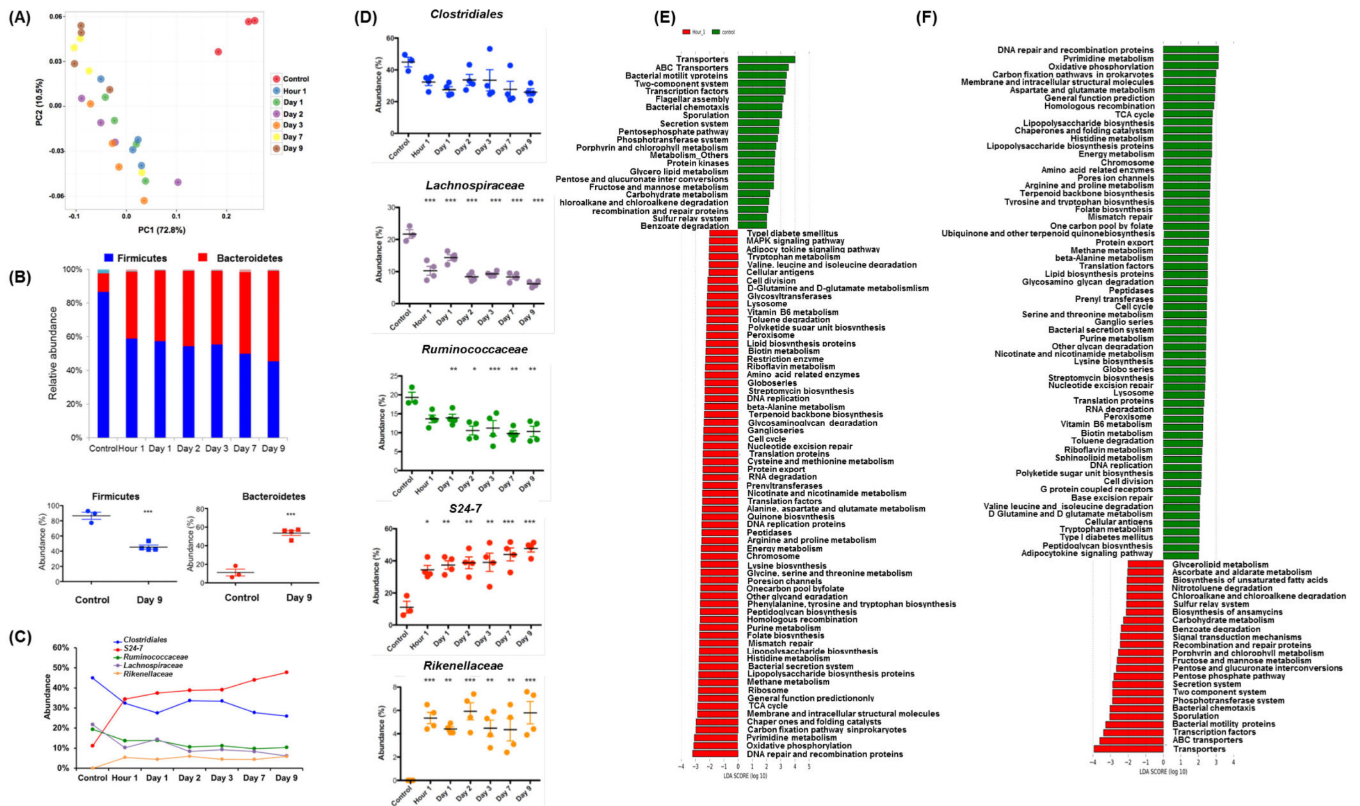

Fig. 1. Partial hepatectomy changed gut microbiota composition

Cecal samples were collected from C57BL/6 mice 0 to 9 days after performing partial hepatectomy (PHx). (A) Principal Coordinates Analysis (PCoA) plot of taxon abundance based on weighted unifrac distance. (B) Bar charts and dot plots representing the composition and changes in Bacteroidetes and Firmicutes during liver regeneration. (C) Most abundant operational taxonomic units (OTUs) changes over time during the course of regeneration. (D) Dot plots show abundance of *Clostridiales* (unidentified family), *S24-7*, *Ruminococcaceae*, *Lachnospiraceae* and *Rikenellaceae* during regeneration. Linear discriminative analysis effect size (LEfSe) of statistically significant KEGG pathways between control and Hour 1 (E) and control and Day 2 (F). Positive LDA scores (green) are enriched in control while negative LDA scores (red) are enriched at Hour 1 and Day 2. Significant differences were determined using ANOVA (*, *p*-value 0.05; **, *p*-value 0.01; ***, *p*-value 0.001).

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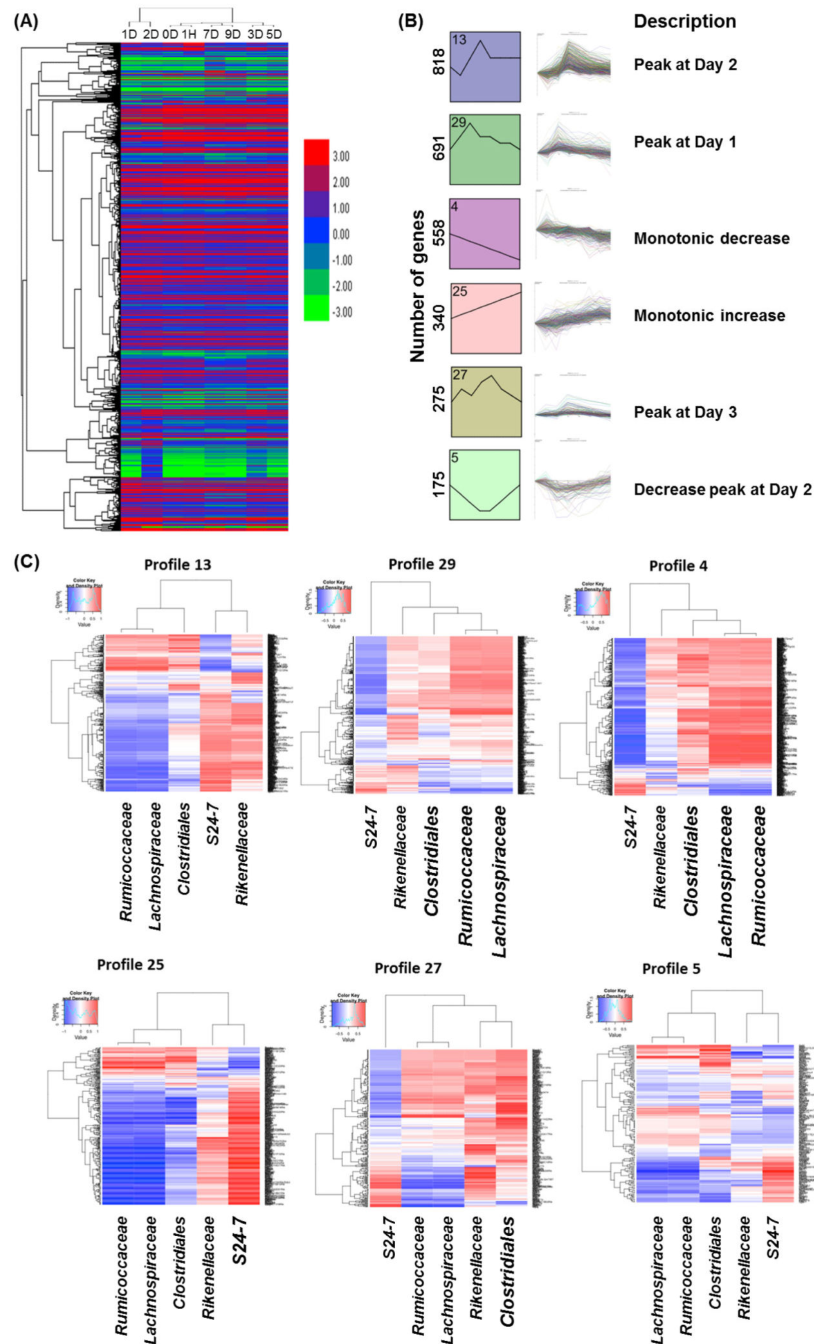

Fig. 2. Hepatic gene expression profiles during liver regeneration

(A) Hierarchical clustering of 6,125 differentially expressed genes during liver regeneration. (B) Identification of 6 significant gene cluster profiles with coherent changes during liver regeneration by short time-series expression miner (STEM) algorithm. (C) Heatmaps of Spearman correlation analysis between abundance of bacterial families and genes present in significant expression profiles.

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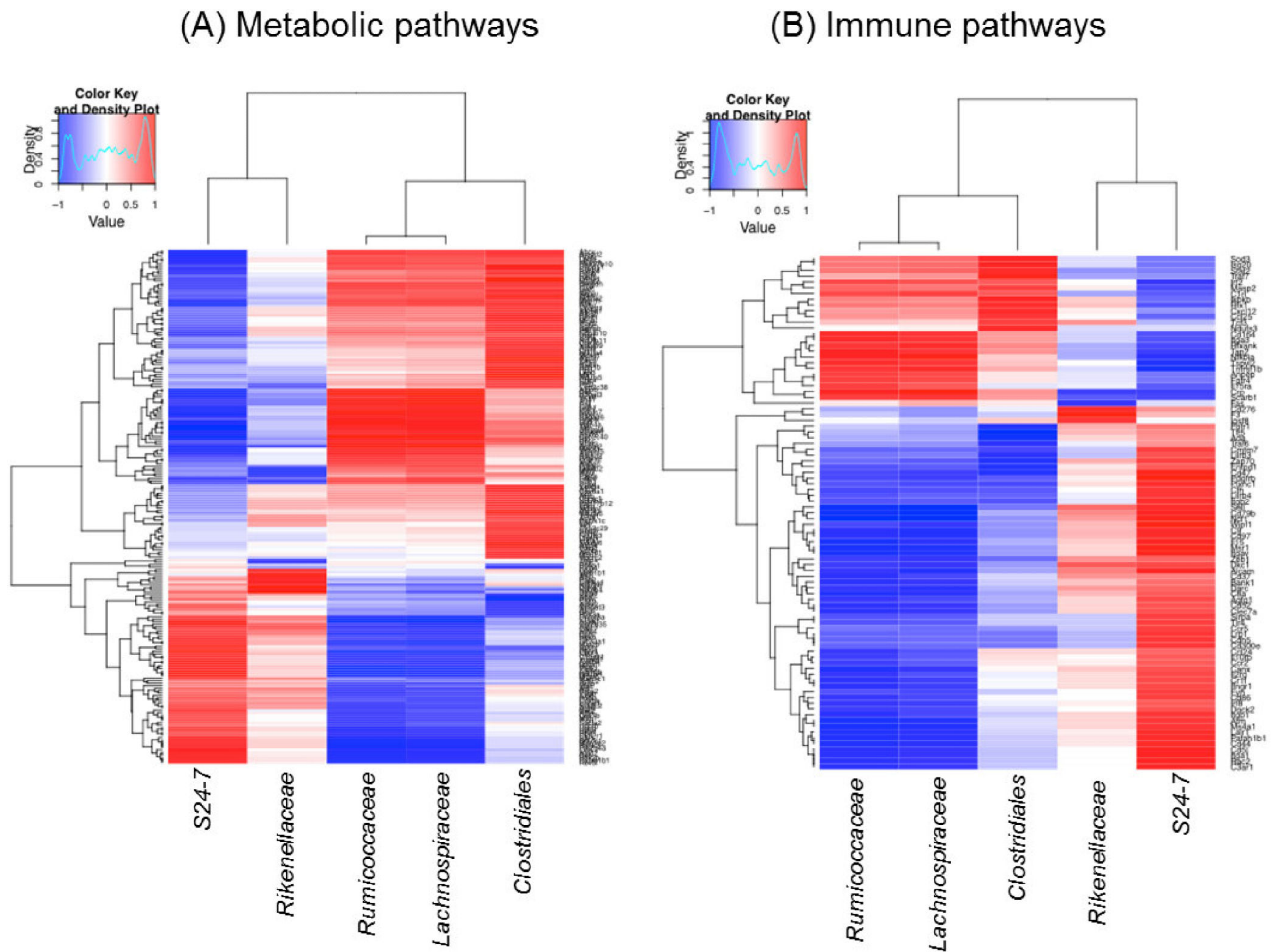

Fig. 3. Spearman correlation analysis

Heatmaps of Spearman correlation analysis between abundance of bacterial families and genes involved in metabolic pathways (A) and immune response (B).

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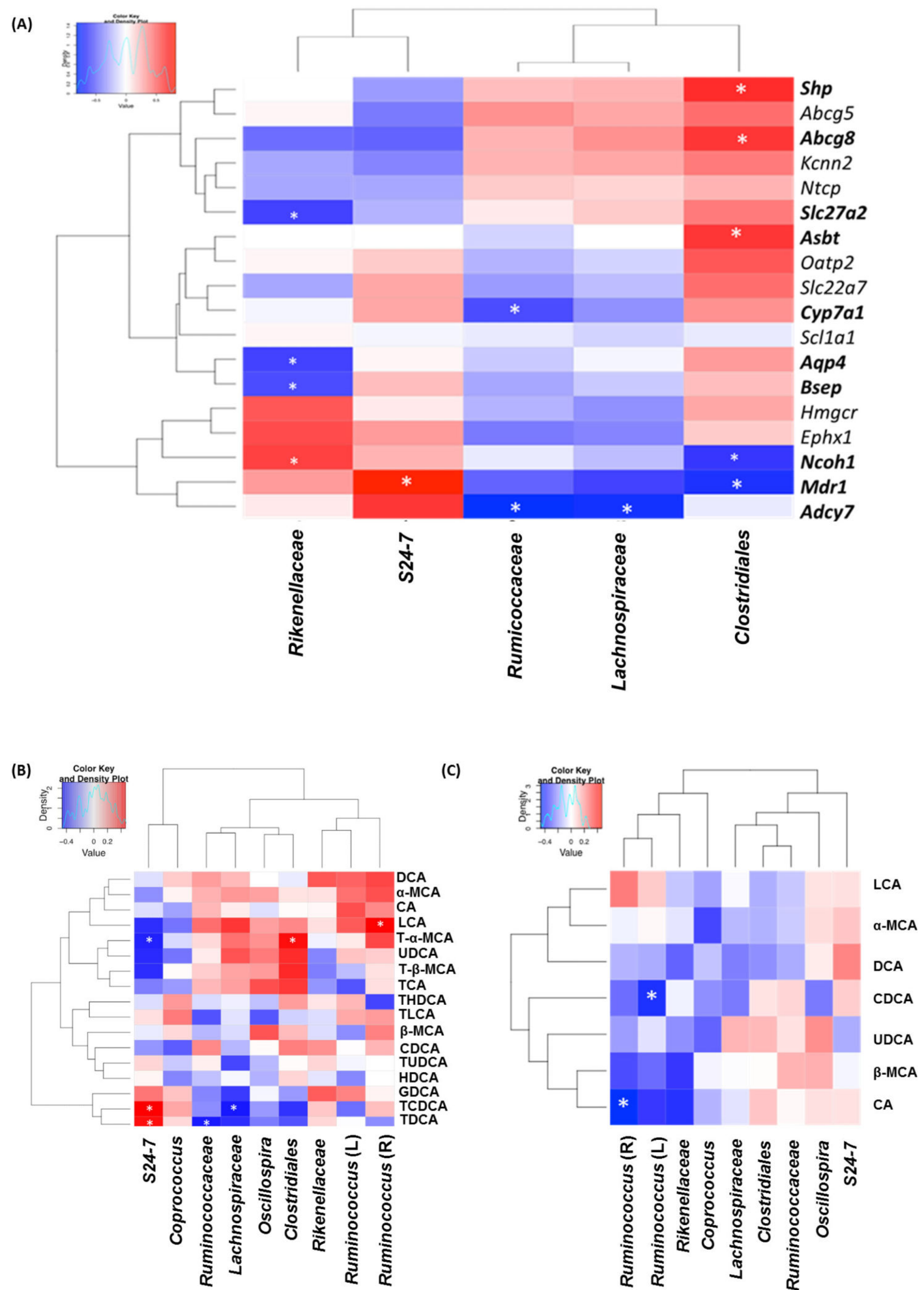

Fig. 4. Interaction between gut microbiota and bile acid pathway during liver regeneration

Heatmaps of Spearman correlation analysis between gut microbiota and hepatic gene expressions involved in bile acid pathways (A), and bile acid profiles in the liver (B) and cecum (C) (*p<0.05).

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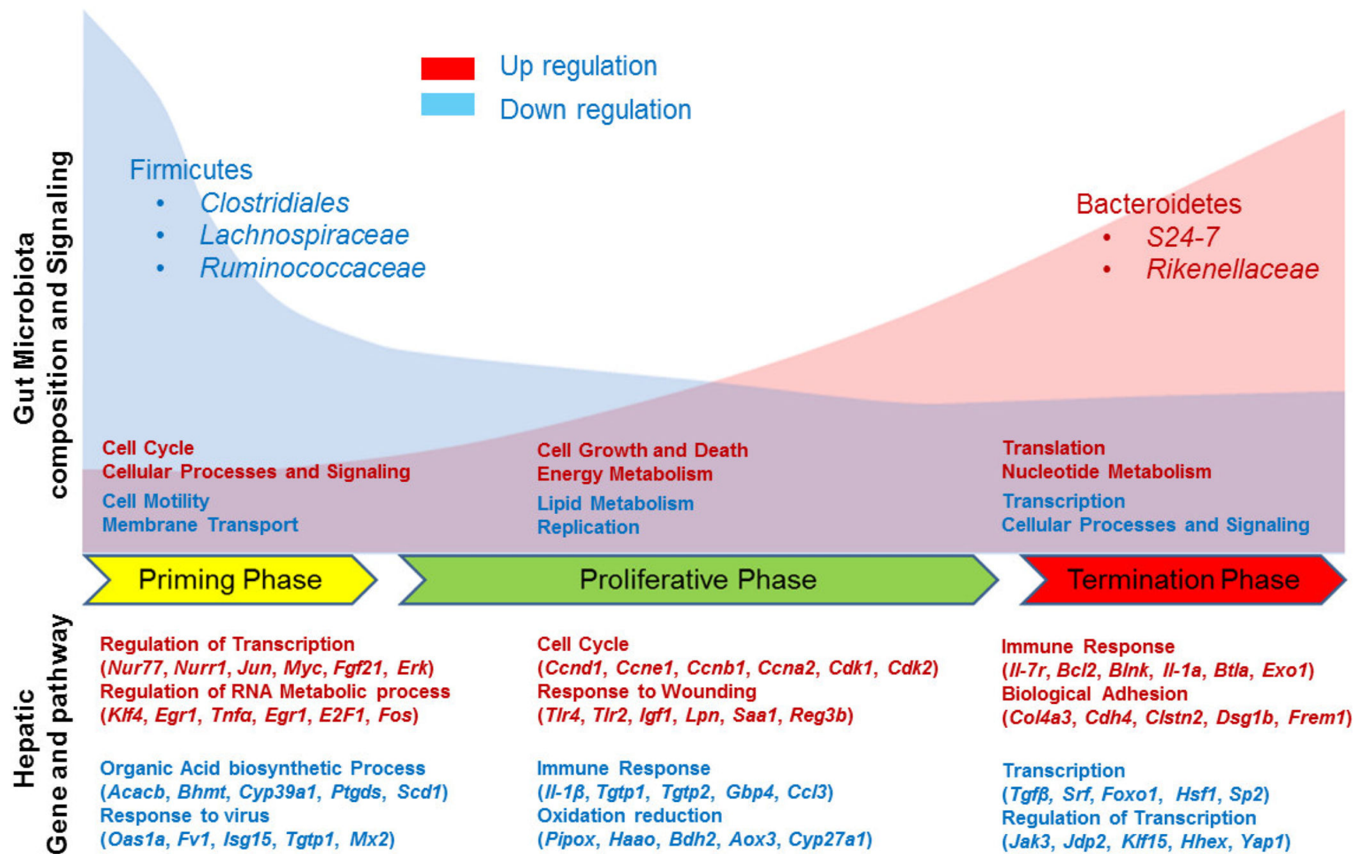

Fig. 5.

The changes of microbiota, metabolites, and pathways during liver regeneration.