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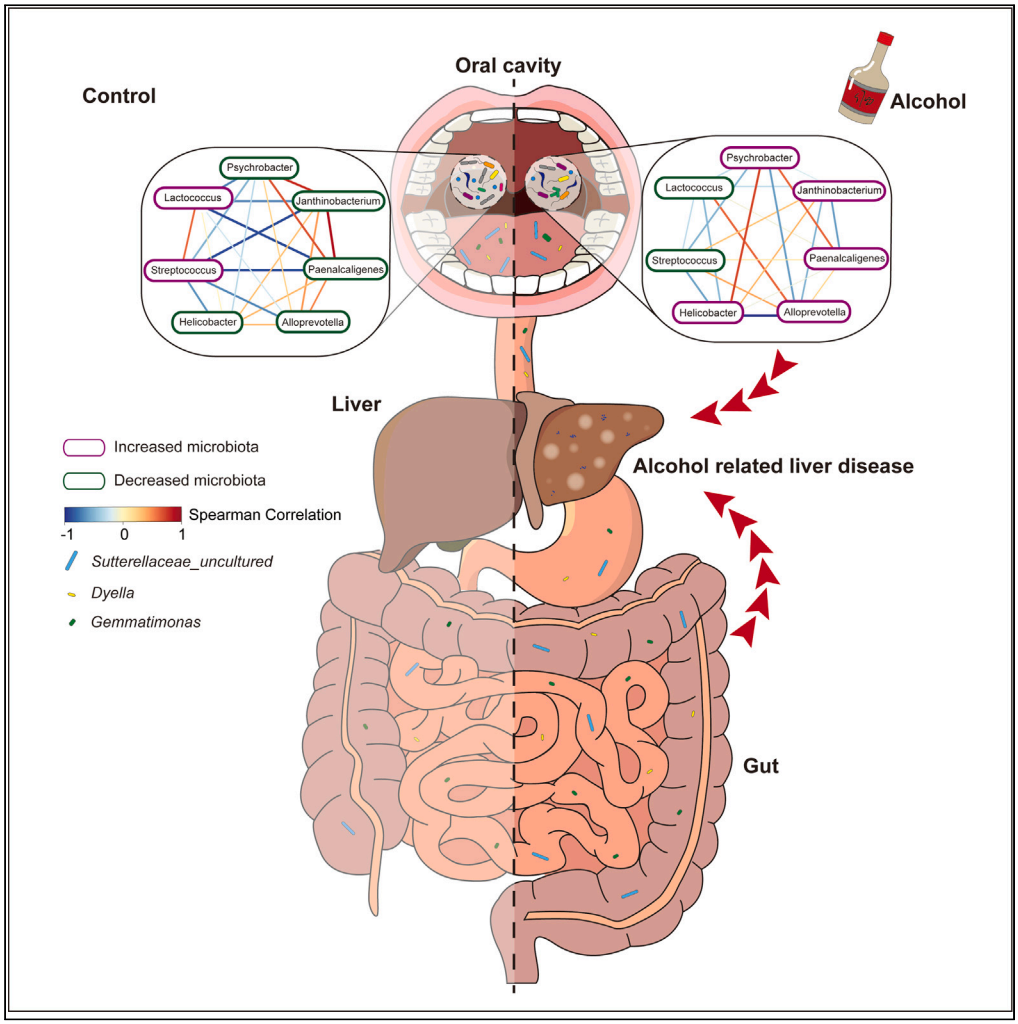

Chuyue Pan, Chang Liu, Wenxin Jia, ..., Xiang Zhu, Maohui Yang, Lirui Wang

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Highlights

Alcohol intake altered oral microbial diversity, composition, and function

Alcohol drinking changes the relationships among oral microbiota

The relative abundance of oral microbiota reflects the severity of ALD

Potential oral-gut bacterial translocation was positively associated with ALD

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Alcohol drinking alters oral microbiota to modulate the progression of alcohol-related liver disease

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SUMMARY

Alcohol-related liver disease (ALD) is one of the leading causes of liver-related death worldwide. However, roles of oral microbiota in regulating the progression of ALD remain unknown. Here, we fed mice with control or ethanol diet to establish chronic-plus-binge ALD model. 16S ribosomal DNA sequencing was performed on oral and cecum samples. We demonstrated that alcohol drinking influenced bacterial richness, microbial structure, and composition in oral samples of ethanol-fed mice compared with control mice. Alcohol consumption also remodeled relationships among oral microbes and altered functions of oral microbiota. Furthermore, oral microbiota, such as *Streptococcus*, *Helicobacter*, *Alloprevotella*, and *Psychrobacter* were closely associated with ALD parameters. Finally, we observed *Sutterellaceae_uncultured*, *Dyella*, and *Gemmatimonas* possibly translocated along with oral-gut axis and positively correlated with the severity of ALD. Altogether, alcohol consumption reprogramed composition and functions of oral microbiota to promote ALD progression, suggesting that oral microbes might become a new target for ALD therapy.

INTRODUCTION

Alcohol-related liver disease (ALD) is one of the most causes of mortality worldwide, with approximately half of the liver cirrhosis-induced death associated with alcohol.[1,2] The spectrum of ALD covers alcoholic fatty liver, alcoholic steatohepatitis (ASH), alcoholic fibrosis, and cirrhosis.[3] Besides, severe ASH may progress to alcoholic hepatitis, in which 20%–50% of patients will die within 3 months.[2,3] Although ALD has been a serious threat to human health, the studies about the pathological mechanism and effective therapeutic approaches are still limited.[2–4] Therefore, it is urgent to elucidate the pathogenesis and explore potential therapeutic strategies for ALD.

With further researches on the pathogenesis of ALD, increasing evidences have demonstrated that alcohol-associated dysbiosis of gut microbiota contributes to ALD progression by various mechanisms, including the disruption of intestinal tight junction, the microbial translocation from gut to the liver, the release of microbial toxins (like lipopolysaccharides), and the metabolism of intestinal bile acids or fatty acids.[5–7] As the second largest microbiome after the gut microbiota, oral microbiota includes more than 700 different species of bacteria, fungi, viruses, and protozoa.[8] The disturbance of oral microbiota results in the increase of pathogenic bacteria, mucosal immunity damage, and the translocation of oral microbiota to other tissues.[9] In consequence, the dysbiosis of oral microbiota is not only associated with oral diseases but also has intimate relationships to systemic diseases, such as liver cirrhosis, liver cancer, pancreatic cancer, inflammatory bowel disease, and cardiovascular disease.[10–13] Although heavy alcohol drinking has been reported to influence the composition of oral microbiota,[14] whether oral microbiota could also take an active role in ALD progression remains unknown.

Here, we demonstrated that the impact of alcohol consumption on oral microbiota during the development of ALD. The microbial diversity was reduced and the microbial structure was changed in the oral cavity of ethanol-fed mice as compared with control mice. The relative abundance of oral *Helicobacter*, *Janthinobacterium*, *Alloprevotella*, *Paenalcaligenes*, and *Psychrobacter* was increased, while *Lactococcus* and *Streptococcus* was decreased following alcohol intake. In addition, we predicted the potential altered functions of oral microbiota during alcohol consumption and analyzed the correlation between the oral microbiota and ALD progression. Finally, further investigation showed that *Sutterellaceae_uncultured*, *Dyella*, and *Gemmatimonas* were probably translocated along with the oral-gut axis, and more importantly, their intestinal abundance was positively associated with ALD. Collectively, our findings have illustrated the critical roles of oral microbiota in promoting ALD progression.

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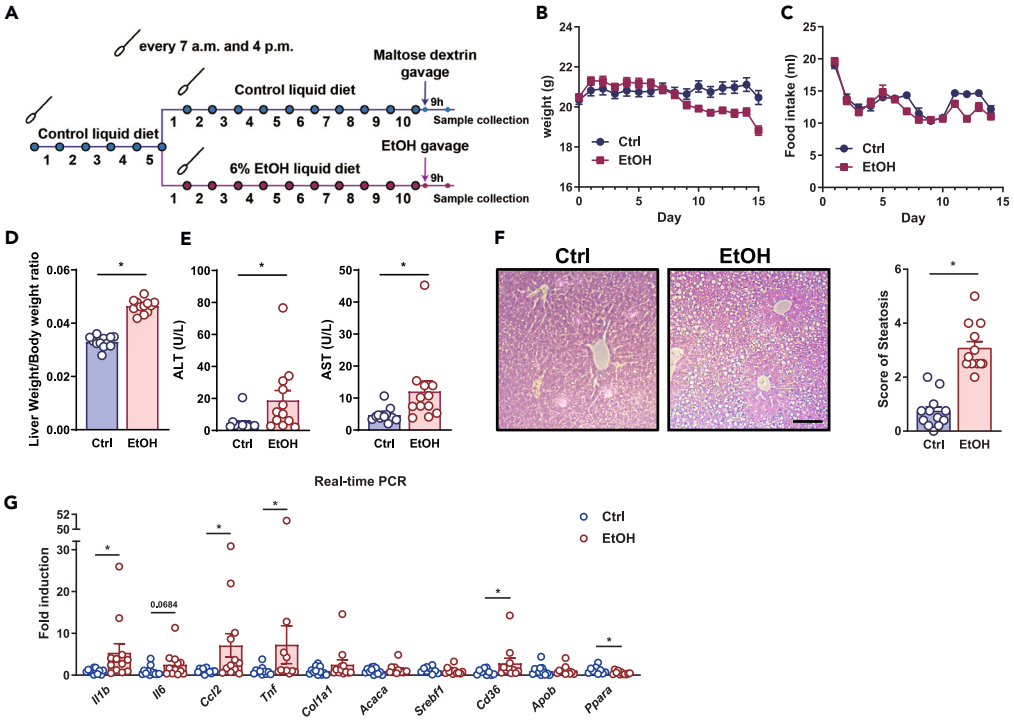

Figure 1. EtOH liquid diet feeding deteriorates the biochemical and histological characters to induce ALD

(A) Schematic of the mice ALD model. Mice were fed with control liquid diet for 5 days, and then diet was changed to 6% EtOH liquid diet in EtOH group for 10 days. At the 15th day, mice were gavaged EtOH or maltose dextrin, respectively. Oral samples were collected every 7:00 a.m. and 4:00 p.m. (n = 12 per group).

(B) Body weight.

(C) Food intake.

(D) Liver weight/body weight ratio.

(E) Plasma ALT and AST levels.

(F) Left: Representative images of H&E staining in liver sections. Scale bar: 100 μm. Right: Scores of steatosis.

(G) Gene expressions of inflammation and lipid metabolism in liver tissues (n = 10–12 per group). Data were presented as mean ± SEM. *p < 0.05. Data were analyzed by two-tailed Student's *t* test except data in G were analyzed by Mann Whitney test.

RESULTS

Alcohol feeding affects the microbial diversity of oral microbiota

Chronic-plus-binge model was utilized to generate mice with ALD (Figure 1A).[15,16] Although alcohol feeding did not affect the body weight and food intake significantly (Figures 1B and 1C), the liver weight/body weight ratio and plasma levels of ALT and AST were markedly increased in ethanol-fed mice (Figures 1D and 1E). Histological staining revealed very obvious hepatic steatosis after alcohol diet feeding (Figure 1F). Compared with mice fed with control diet, the real-time PCR data showed the elevated mRNA expression of inflammatory cytokines, such as *Il1b*, *Il6*, *Ccl2*, and *Tnf*, in the livers of ethanol-fed mice. Gene-encoding fatty acid translocase (*Cd36*) was also upregulated, while *Ppara*, the gene responsible for fatty acid β-oxidation was decreased following alcohol dinking (Figure 1G). Taken together, alcohol feeding could induce severe hepatocytes death, hepatic steatosis, and inflammation in livers of mice.

To investigate the effects of alcohol feeding on the oral microbiota and assess whether this impact will be different in the morning or in the evening, oral samples were collected daily at 7:00 a.m. and 4:00 p.m. from control and ethanol-fed mice (Figure 2A). α-diversity which represents species richness and evenness was shown by Chao 1, Shannon, and Simpson indexes. In spite of the less impact of alcohol drinking on samples collected in the morning from the oral cavity of mice (Figure 2B), oral samples collected in the evening showed obvious decrease of microbial diversity following alcohol intake (Figure 2C). We also compared the α-diversity of oral samples collected in the morning or in the evening and the cecum samples. As expected, we found that the microbial richness was quite higher in the gut microbiota than that in oral cavity of both control and ethanol groups (Figure 2D). Moreover, in mice fed with ethanol diet, the oral bacterial richness was significantly lower in samples collected in the evening than that in the morning as evidenced by Chao 1 index and Shannon index (Figure 2D), while in control diet-fed mice, the oral bacterial diversity was not changed between these two time points (Figure 2D). The compositional dissimilarity among groups was described by β-diversity. We firstly observed a significant difference in clustering between the gut microbiota of control and ethanol-fed mice ($p = 0.018$) (Figure 2E). Further, the β-diversity analysis revealed an obvious separation between control and ethanol diet-fed mice in oral samples collected in both the morning ($p = 0.018$) (Figure 2F, Left panel) and evening ($p = 0.022$) (Figure 2F, Right panel).

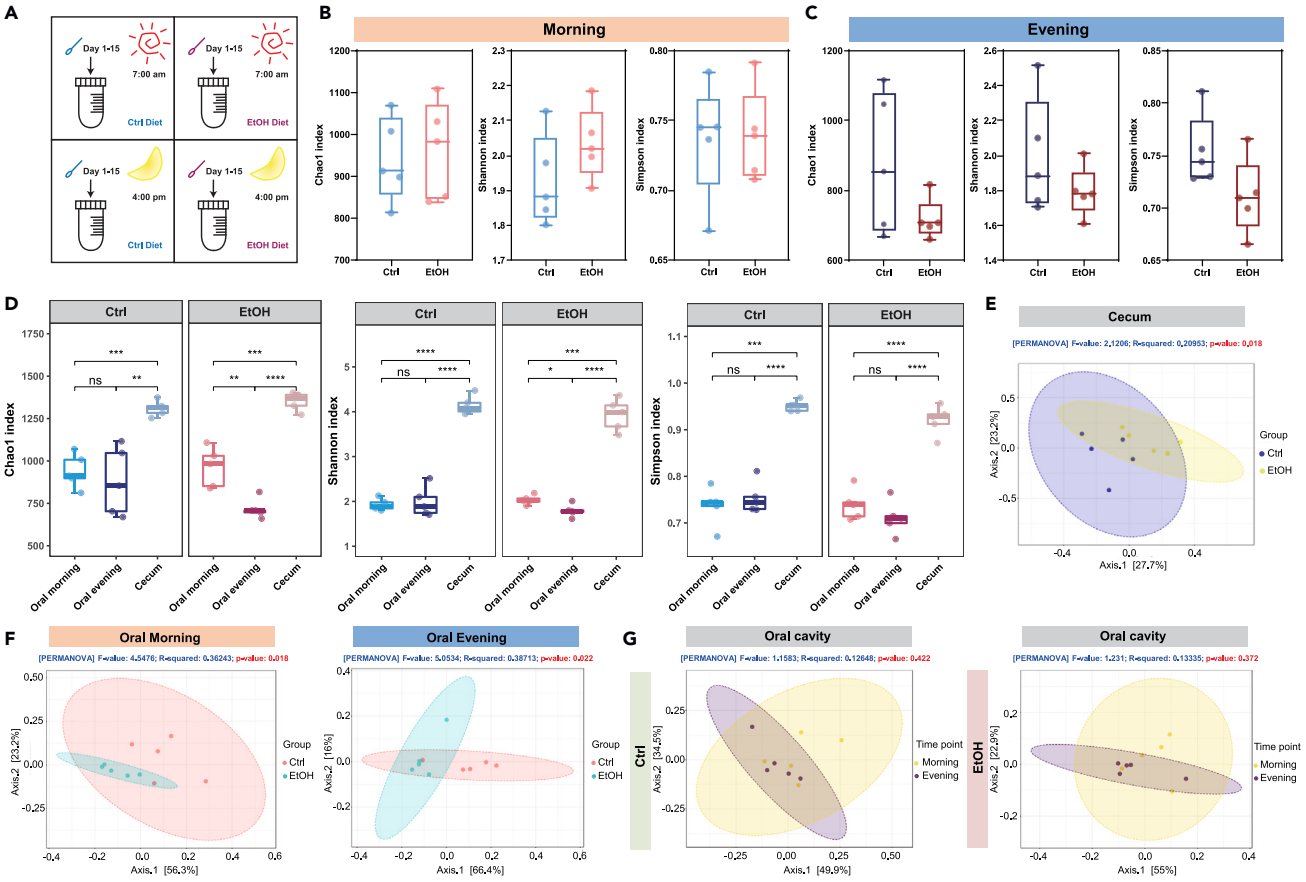

Figure 2. Alcohol consumption affects the microbial diversity of oral microbiota

(A) Schematic of the samples collecting.

(B and C) The α-diversity was showed by Chao 1 index, Shannon index, and Simpson index. Oral samples were collected from mice fed with control and EtOH diet in the morning (B) or in the evening (C).

(D) Comparison of the α-diversity of oral samples collected in the morning or in the evening and the cecum samples from mice fed with control and EtOH diet.

(E) β-diversity in cecum microbiota of Ctrl and EtOH groups.

(F) β-diversity of control group and EtOH group in oral samples collected in the morning (Left) and in the evening (Right).

(G) The β-diversity between samples collected in the morning or in the evening in control (Left) and ethanol-fed mice (Right). *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.001.

Of note, we have investigated the microbial structure in oral samples collected in the morning and in the evening and found that microbiota composition in samples collected in the morning was not clustered with that in samples collected in the evening in control group (p = 0.422) (Figure 2G, Left panel) and ethanol group (p = 0.372) (Figure 2G, Right panel). Overall, alcohol feeding altered the microbial richness and structure of oral microbiota especially in samples collected in the evening.

Alcohol consumption alters the composition of oral microbiota

Since that oral samples collected in the evening showed the most significant differences in microbial diversity in control and ethanol-fed mice, we thus chose these oral samples for further analysis on microbial composition. The relative abundance of oral microbiota in phyla levels was described using bar charts (Figure 3A). We have compared the oral microbial composition between mice fed with control and alcohol diet, and observed that at the phylum level, the relative abundance of Proteobacteria was obviously elevated in ethanol-fed mice compared with control mice, while Firmicutes, Acidobacteriota, Chloroflexi, Myxococcota, and *RCP2-54* was significantly decreased after alcohol feeding (Figures 3A–3C). At the level of genus, the bar charts showed the remarkable upregulation in the amount of *Janthinobacterium*, *Psychrobacter*, and *Paenalcaligenes* in the oral samples of ethanol-fed mice. In contrast, we found that the relative abundance of *Streptococcus* was significantly reduced in the oral cavity of alcohol-consumed mice, and there was also a decreased tendency in the abundance of *Pseudomonas*, *Lactococcus*, and *Acinetobacter* (Figures 3D–3F).

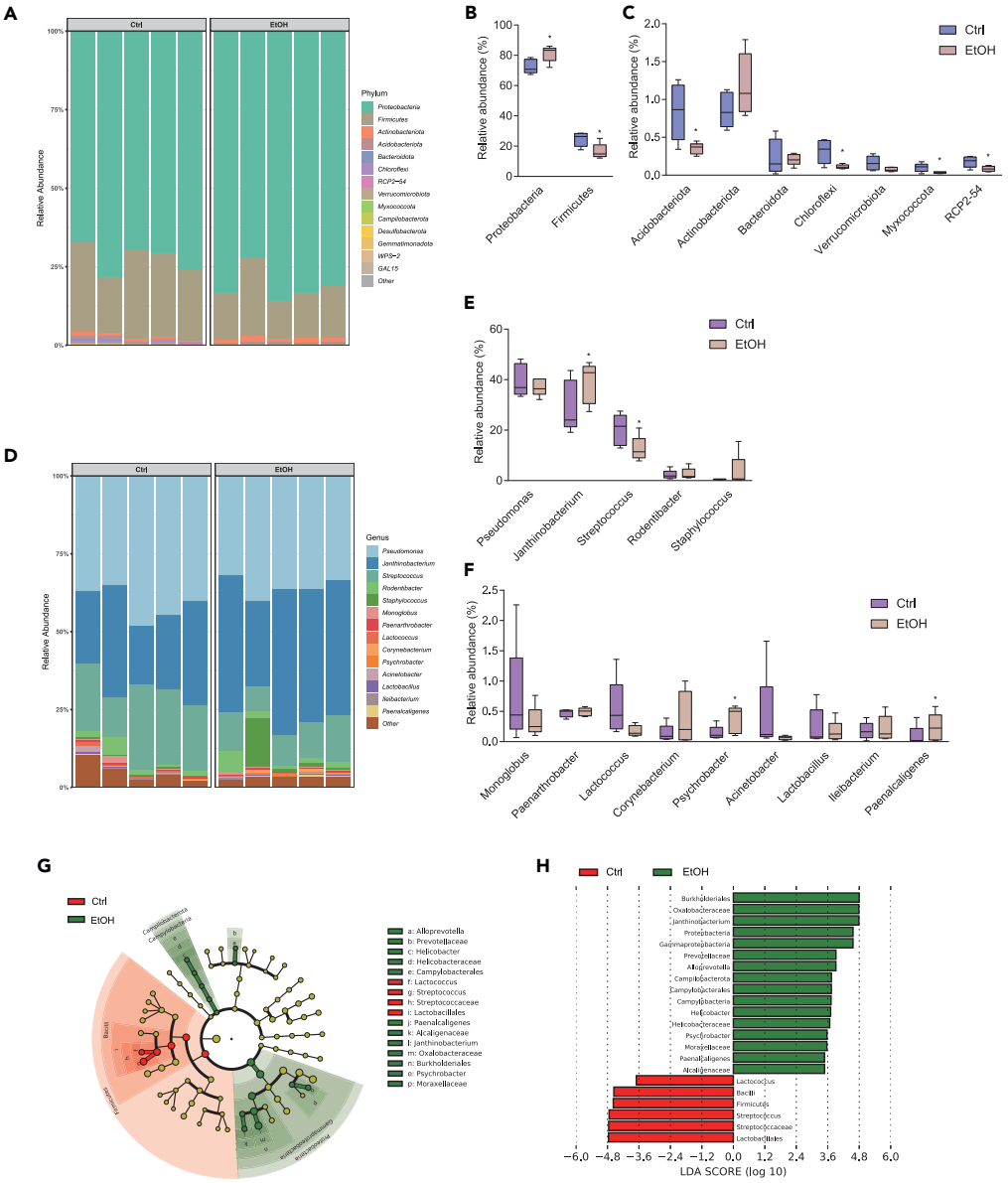

Figure 3. Alcohol drinking changes the microbial community structures in oral cavity

(A–C) The relative abundance of oral bacterial at phylum level. (A) Top 14 phyla were presented in order of their relative abundance in the bar chart and the rest was assigned as "others". (B) The relative abundance of *Proteobacteria* and *Firmicutes*. (C) The relative abundance of Acidobacteriota, Actinobacteriota, Bacteroidota, Chloroflexi, Verrucomicrobiota, Myxococcota, and *RCP2-54* in the oral cavity. Data were presented as mean ± SEM. *p < 0.05.

(D–F) The relative abundance of oral microbiota at genus level. (D) Bar chart of the top 14 genera in order of their relative abundance. (E) The relative abundance of top 5 genera with abundance >5%. (F) The relative abundance of other 9 genera. Data were presented as mean ± SEM. *p < 0.05.

(G) The cladogram of oral microbiota in different taxonomic levels. Red and green represented the differential taxa from control and ethanol-fed mice, respectively (n = 5 per group) (Wilcoxon rank-sum test, p < 0.05).

(H) Significantly changed bacterial taxa between control and ethanol group of mice (LDA score, >2 or < −2). Data were analyzed by two-tailed Student's *t* test in B, C, E, and F.

Consistently, the linear discriminant analysis effect size (LEfSe) analysis of oral microbiota in control and ethanol-fed mice also demonstrated that ethanol intake significantly increased the relative abundance of Campilobacterota and Proteobacteria at the phylum level and dramatically elevated the relative abundance of *Helicobacter*, *Janthinobacterium*, *Alloprevotella*, *Paenalcaligenes*, and *Psychrobacter* at the genus level, while genus of *Lactococcus* and *Streptococcus* were obviously decreased in the oral cavity following alcohol feeding (Figures 3G and 3H). Altogether, we revealed that alcohol consumption significantly upregulated the abundance of *Janthinobacterium*, *Paenalcaligenes*, and *Psychrobacter* and remarkably reduced the relative abundance of *Streptococcus* and *Lactococcus* in oral cavity.

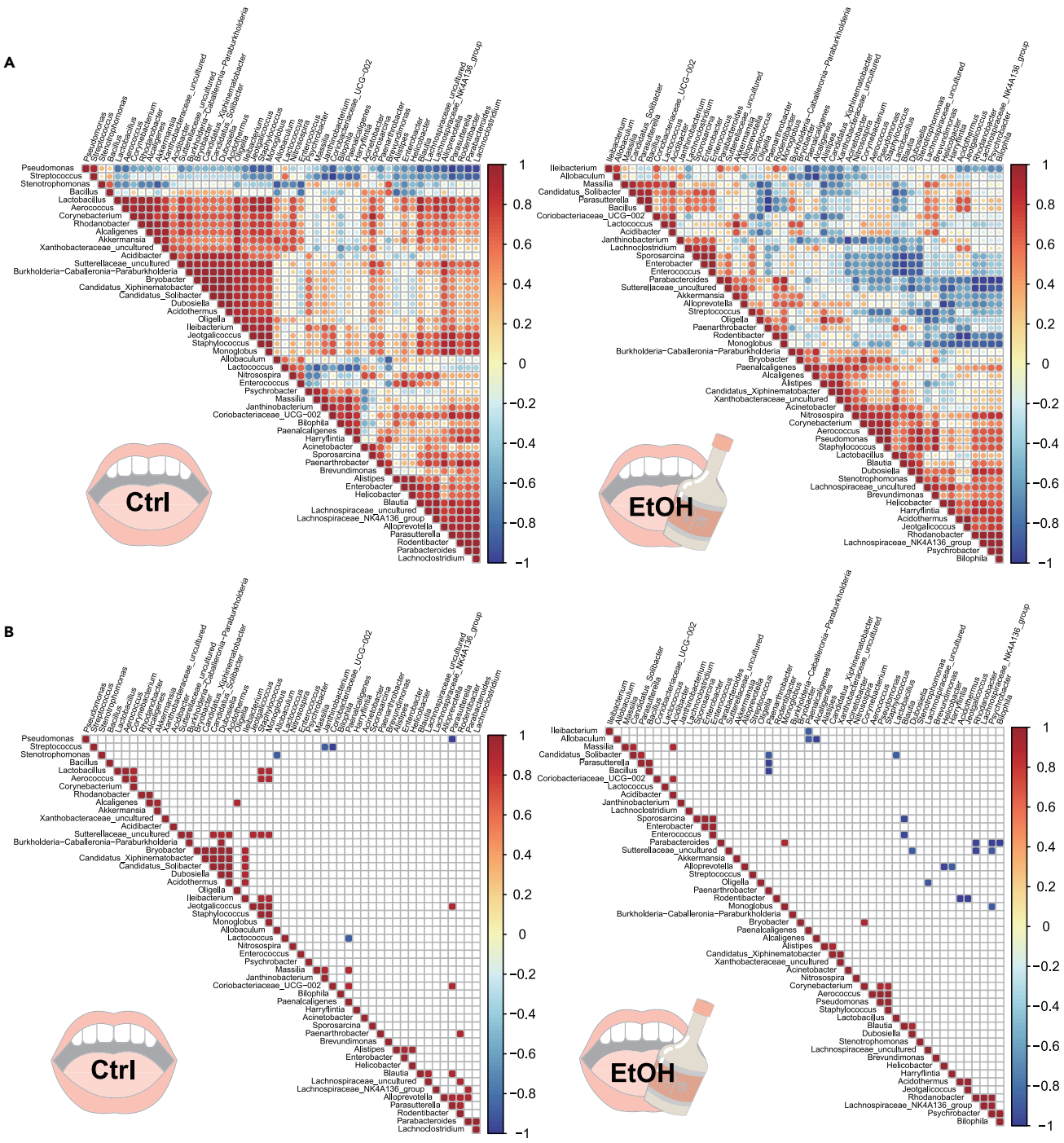

Figure 4. Ethanol diet feeding reprograms the correlation between oral microbiota

(A) Correlation between oral bacteria under control diet (Left) or ethanol diet (Right) feeding.

(B) The significant relationships between oral microbes ($p < 0.05$) were selected to be shown.

Ethanol diet reprograms the relationships among oral microbiota

Apart from providing information about taxonomic composition of oral microbiota, we further analyzed the relationships among microbiota in mice fed with control or ethanol diet. In the oral cavity of control mice, most microbes displayed positive interactions with each other (Figures 4A and 4B, Left panels), but following alcohol feeding, we observed that a larger proportion of microbiota shifted to behave

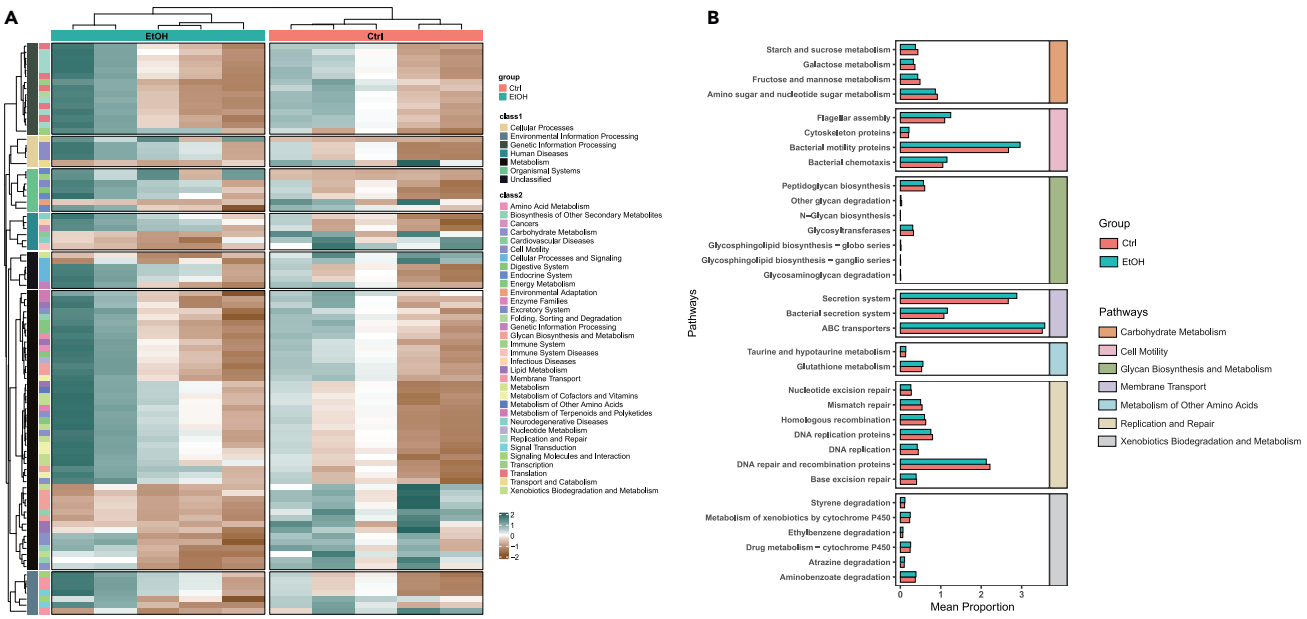

Figure 5. Effects of alcohol on the functional profile of oral microbiota

(A) The prediction of functional profiles by 16S rRNA sequencing analysis of oral microbiota in control and EtOH group of mice.

(B) The upregulated and downregulated pathways influenced by alcohol consumption.

negatively correlated with other bacteria as evidenced by more blue-color area in the correlation plots (Figures 4A and 4B, Right panels). Specifically, *Monoglobus* was positively correlated with *Lactobacillus*, *Aerococcus*, *Sutterellaceae_uncultured*, *Ileibacterium*, *Jeotgalicoccus*, and *Staphylococcus* in control mice (Figure 4B, Left panel), while it was only positively correlated with *Parabacteroides* after alcohol feeding (Figure 4B, Right panel). *Acidothermus* and *Dubosiella* were both positively correlated with *Candidatus_Xiphinematobacter*, *Sutterellaceae_uncultured*, *Bryobacter*, and *Candidatus_Solibacter*, but these correlations were disappeared after alcohol feeding. These results indicated that alcohol drinking disrupted the relationships among oral microbiota. Meanwhile, we found that new correlations appeared following alcohol feeding. For example, *Paenarthrobacter* almost failed to correlate with other microbiota in the oral cavity of control mice, while *Paenarthrobacter* was inversely correlated to *Candidatus_Solibacter*, *Parasutterella*, and *Bacillus* following alcohol feeding. Notably, alcohol reprogramed the connections of *Psychrobacter* with other bacteria, making it become the busiest hub in ethanol-fed mice, while *Psychrobacter* had no remarkable correlations with other bacteria under the control diet feeding (Figure 4B). Collectively, alcohol consumption remodeled the relationships among microbiota in the oral cavity.

Effects of alcohol on the function of oral microbiota

We further predicted the functions of oral microbiota in control and ethanol-fed mice, and the significant altered pathways were demonstrated in Figure 5A. More specifically, among the pathways which were decreased after alcohol feeding, a large part of them were associated with carbohydrate metabolism and glycan biosynthesis and metabolism, including pathways of galactose metabolism, glycosaminoglycan degradation, starch and sucrose metabolism, fructose and mannose metabolism, and so on (Figure 5B, orange and green panels). We also noticed that alcohol feeding decreased the DNA replication and repair pathway in ethanol-fed mice, indicating that alcohol consumption damaged the survival of bacteria (Figure 5B, yellow panel). In contrast, alcohol upregulated the pathways related to alcohol metabolism, such as glutathione metabolism and metabolism by cytochrome P450 (Figure 5B, blue and gray panels). We next revealed that alcohol feeding also increased pathways of microbial secretion system, suggesting that alcohol might promote the communication between oral microbiota and the host cells (Figure 5B, purple panel). Finally, the upregulation of pathways about bacterial motility proteins, bacterial chemotaxis, and flagellar assembly in alcohol-fed mice implied that oral microbiota might have higher capability to translocate from oral cavity to internal tissues following alcohol feeding (Figure 5B, pink panel).

The relative abundance of oral microbiota reflects the severity of ALD

To investigate whether the change of the oral microbiota after alcohol feeding was associated with ALD progression, we analyzed the relationships of the top 50 abundant oral bacteria with ALD symptoms including ALT, AST, steatosis, and hepatic mRNA levels of inflammation and lipid metabolism (Figure 6A). Among them, 24 genera were significantly correlated with these ALD phenotypes; their Spearman correlations were described in Figure 6B. The relative abundance of *Helicobacter*, *Oligella*, *Alcaligenes*, *Psychrobacter*, and *Alloprevotella* was positively correlated with ALT, AST, and hepatic steatosis. In contrast, we found that the relative abundance of *Streptococcus*, *Lactococcus*,

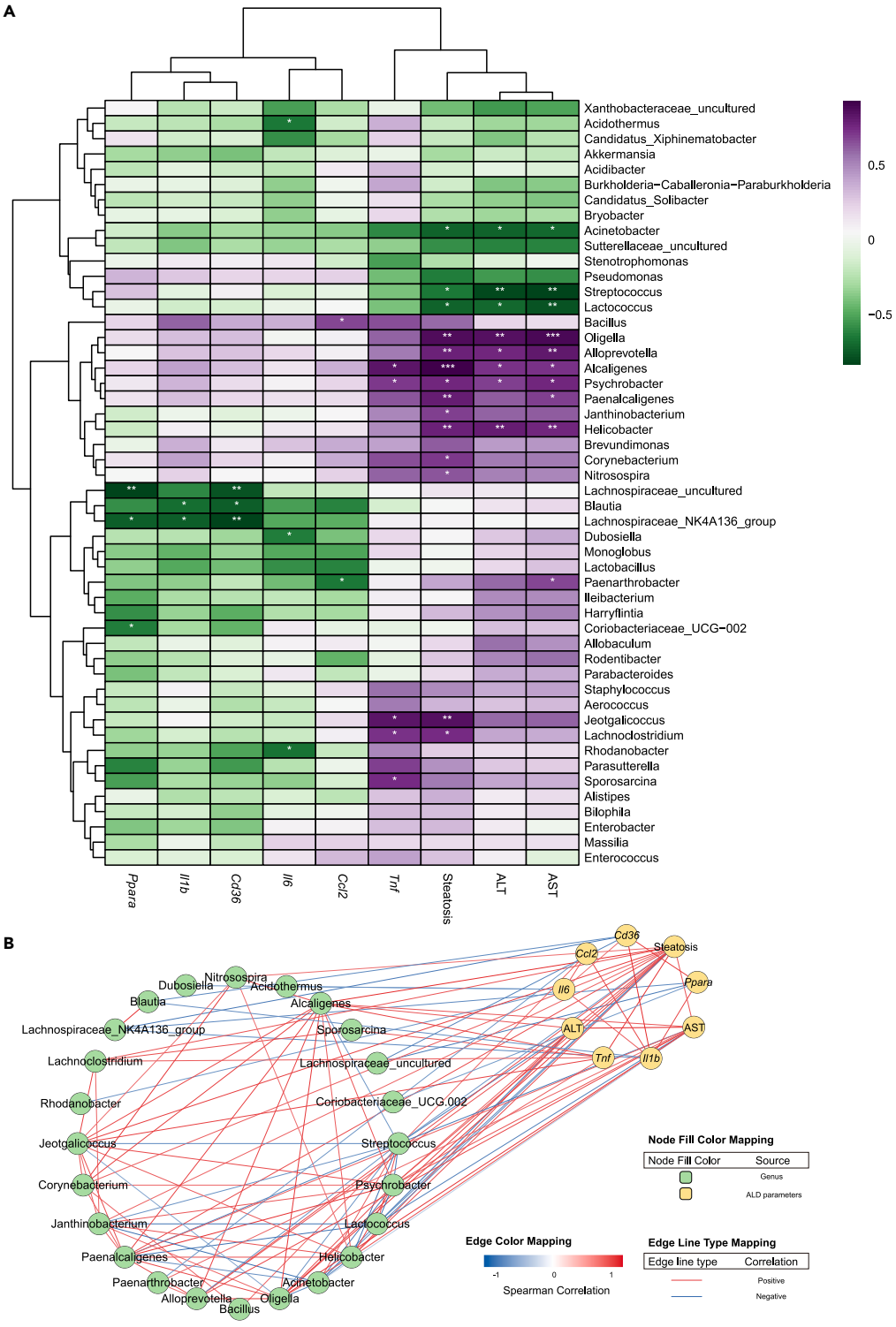

Figure 6. Oral microbiota is associated with ALD progression

(A) Heatmap of Spearman's correlation among top 50 genera in the oral cavity and several ALD parameters. *p < 0.05, **p < 0.01, ***p < 0.001.

(B) Correlation network of the significant (p < 0.05) related nodes. Colors of edge represent Spearman's correlation coefficients, and colors of nodes for their categories.

and *Acinetobacter* showed negative relationships with the hepatic ALT, AST, and steatosis levels (Figures 6A and 6B). Additionally, the abundance of *Blautia* and *Lachnospiraceae_NK4A136_group* was negatively correlated with the expression of *Il1b* in liver tissues. The alteration of *Acidothermus*, *Rhodanobacter*, and *Dubosiella* was revealed to be inversely correlated with hepatic *Il6* expression, and the relationship of *Paenarthrobacter* abundance with *Ccl2* mRNA level was negative as well. We also observed that the abundance of *Psychrobacter*, *Jeotgalicoccus*, and *Alcaligenes* had positive relationships with the hepatic *Tnf* level (Figures 6A and 6B). Finally, we noticed that the relative abundance of *Lachnospiraceae_uncultured*, *Blautia*, and *Lachnospiraceae_NK4A136_group* was dramatically negatively correlated with the expression of fatty acid translocase (*Cd36*) and *Ppara*, suggesting that this microbiota might contribute to liver steatosis (Figures 6A and 6B). Collectively, the reprogramming of oral microbiota by alcohol drinking possibly indicated the development of ALD.

Alcohol induces the oral-intestine translocation of microbiota

Bacterial translocation from oral cavity to other internal tissues was one of the major routes for oral microbiota to influence systemic diseases.[10–13] Considering that gut microbiota has been reported to regulate ALD progression,[5–7] we investigated whether oral bacteria could translocate to the intestine and take part in modulating ALD development. Through comparing of oral microbiota with gut microbiota in control and ethanol-fed mice, we demonstrated that the relative abundance of *Sutterellaceae_uncultured*, *Dyella*, and *Gemmatimonas* was decreased in oral cavity and increased in cecum after ethanol diet feeding (Figure 7A). We further revealed that the relative abundance of *Sutterellaceae_uncultured*, *Dyella*, and *Gemmatimonas* in the oral cavity exhibited negative correlations with alcoholic liver phenotypes including ALT, AST, steatosis, inflammation, and lipid metabolism-related genes, while in the gut, the abundance of these three bacteria was positively correlated with the previously described ALD symptoms (Figure 7B), suggesting that the shift of microbiota from oral cavity to gut might promote the ALD progression. Notably, the elevated relative abundance of *Sutterellaceae_uncultured* in intestine showed significant positive correlations with the hepatic levels of ALT, AST, and steatosis, as evidenced by the linear regression analysis in Figure 7C. Taken together, we found that oral microbiota possibly translocated along with the oral-gut axis to the intestine, and their enrichment in the intestine might promote the progression of ALD.

A machine learning prospective model predicts ALD development

Based on the oral microbiota which was found to be associated with alcohol drinking, we established a noninvasive risk assessment model to estimate whether mice developed ALD or not. This predictive model was created using 5 increased oral microbiota (*Helicobacter*, *Janthinobacterium*, *Alloprevotella*, *Paenalcaligenes*, and *Psychrobacter*) and 2 downregulated oral microbiota (*Lactococcus* and *Streptococcus*), as well as *Sutterellaceae_uncultured*, *Dyella*, and *Gemmatimonas* which had possibility to translocate from oral cavity to gut following alcohol drinking (Figure 8A). According to the results of 16S rRNA sequencing of oral microbiota collected in the evening from mice fed with or without alcohol drinking in this study, we calculated that this predictive model achieved a performance with the area under the receiver operating characteristic curve of 0.78 (Figure 8B), indicating that the discriminatory ability of this model was acceptable.[15] However, the suitability of this model for clinical usage needs further verification, since that the samples' number used here to establish the predictive model was very limited.

DISCUSSION

ALD contributes markedly to the global morbidity and mortality of liver diseases.[2,16] Given the complexity of ALD pathogenesis, the clinical diagnostic and therapeutic strategies are still limited.[6] Increasing evidences have showed the importance of oral microbiota in systemic diseases;[17,18] however, the detailed connection of oral microbiota with ALD has not been well described.[14,19] Therefore, here we performed 16S rRNA sequencing on the oral microbiota of chronic-plus-binge-induced ALD mice to investigate the changes of oral microbiota forced by alcohol intake. We demonstrated that alcohol feeding altered the microbial diversities and compositions as well as functions of oral microbiota in ethanol-fed mice as compared with control mice. The relationships among oral microbiota were also changed following alcohol feeding. Notably, we observed the significant correlations between the alterations of oral microbiota and ALD progression after ethanol intake. We further identified that three genera of bacteria, *Sutterellaceae_uncultured*, *Dyella*, and *Gemmatimonas*, might shift along with the oral-gut axis to the intestine to promote the development of ALD (Figure 8C). Consistent with our findings, several studies also demonstrated that human participants with the long-term alcohol drinking habit or alcohol dependence had the altered oral microbiota compositions and metabolisms.[14,19,20] Although there is no report up to today about the roles of oral microbiota in ALD patients in clinics, our results and the previously mentioned studies indeed shed new light on the potential important functions of oral microbiota in regulating ALD progression in human patients, which merits further investigation.

Since evidences showed that saliva microbiome was influenced by the diurnal rhythm in patient with alcohol dependence,[20] we thus collected oral samples from control and ethanol-fed mice at 7:00 a.m. and 4:00 p.m. daily to investigate the differences of oral microbiota in the morning and in the evening. In our study, we observed a slight increase of α-diversity after alcohol feeding in oral samples collected in the morning and significant decrease of microbiota diversity in oral samples collected in the evening, whereas previous report on saliva microbiota in people with alcohol drinking showed an obvious increase in microbial richness at 7:00 a.m. or at 3:00 p.m.[20] This discrepancy might be due to the fact that experiment on human samples involved much more variables, including diet, smoking, diseases, and other confounding factors. However, our investigation on mice was able to exhibit a more direct impact of alcohol feeding on oral microbiota. Further, although the effects of alcohol intake on oral microbiota were not obviously different between samples collected in the morning and in the

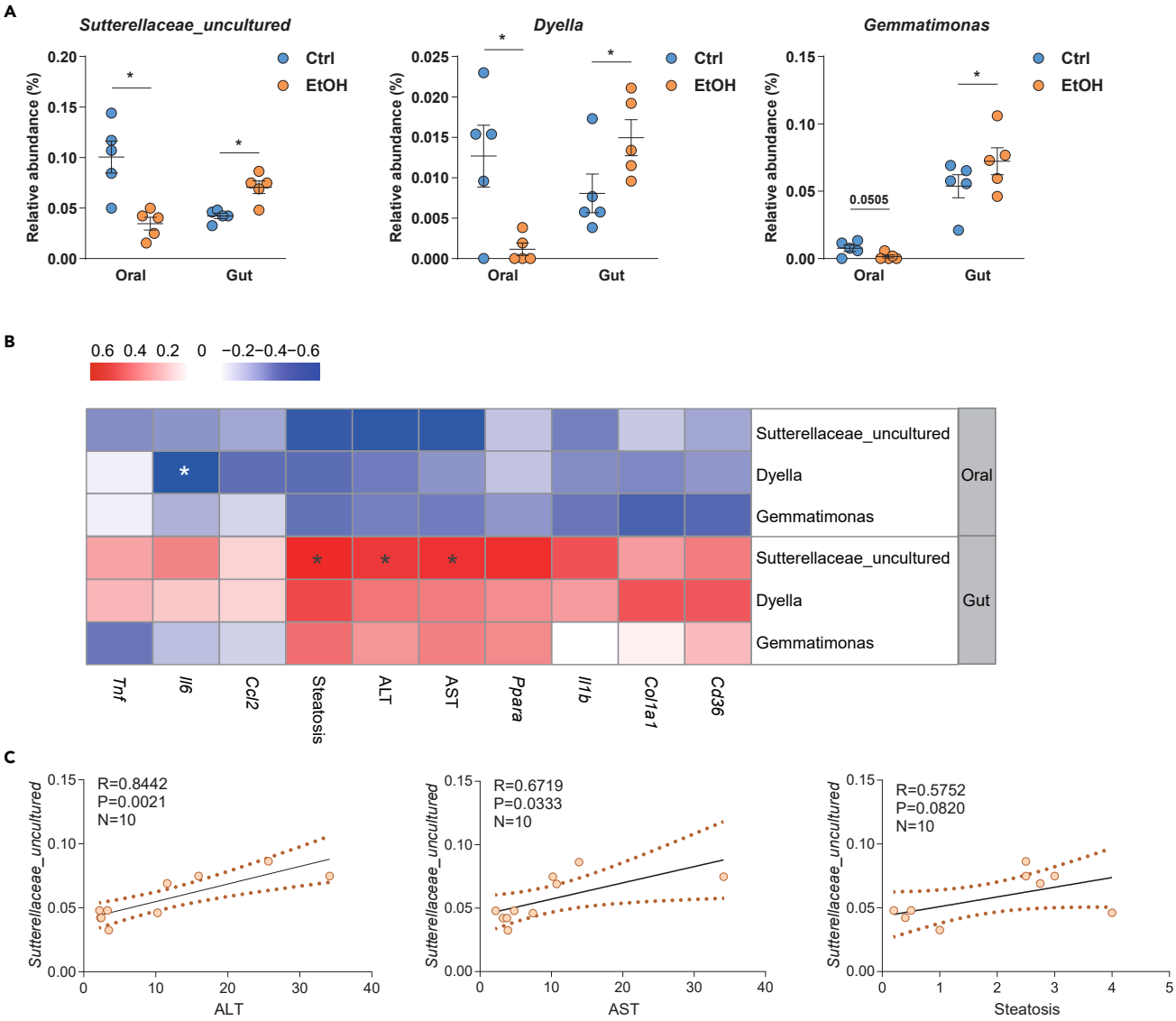

Figure 7. Ethanol consumption induces oral-intestine translocation of oral microbiota

(A) The relative abundance of *Sutterellaceae_uncultured*, *Dyella*, and *Gemmatimonas* in oral and cecum samples of mice fed with control and EtOH liquid diet.

(B) Correlations among abundance of *Sutterellaceae_uncultured*, *Dyella*, *Gemmatimonas*, and ALD symptoms.

(C) Linear regression of the relative abundance of *Sutterellaceae_uncultured* with ALT, AST, and steatosis level. Data were presented as mean ± SEM. *p < 0.05. All data were analyzed follow the procedure of statistical analysis.

evening according to the analyses about microbial structure, we still found that alcohol had affected much more on oral samples collected in the evening, indicating that the changes of alcohol intake on oral microbiota were indeed influenced by diurnal rhythm in some degree.[20]

In this study, we observed significant increases in the relative abundance of *Janthinobacterium* and *Helicobacter* after alcohol feeding in the oral cavity, while the abundance of *Streptococcus* was obviously decreased. It was reported that oral *Helicobacter* may promote periodontal disease, and then induce the release of oral inflammatory cytokines.[21] Moreover, the relationship between periodontal disease and liver diseases has also been revealed.[9] These might be consistent with our findings that the elevated *Helicobacter* in the oral cavity was positively correlated with ALD parameters. In addition, *Streptococcus* is the dominant species of oral cavity, whose abundance may be related to the pH level of oral cavity.[22] The abundance of certain acid-nonresistant *Streptococcus* might be decreased due to the influence of organic acids produced by alcohol metabolism.[22] Further, evidences showed that *Janthinobacterium* played important roles in proteolysis, lipolysis, and saccharolysis,[23] while we have no clear clue about the function of *Janthinobacterium* in the oral cavity, which might need further investigations.

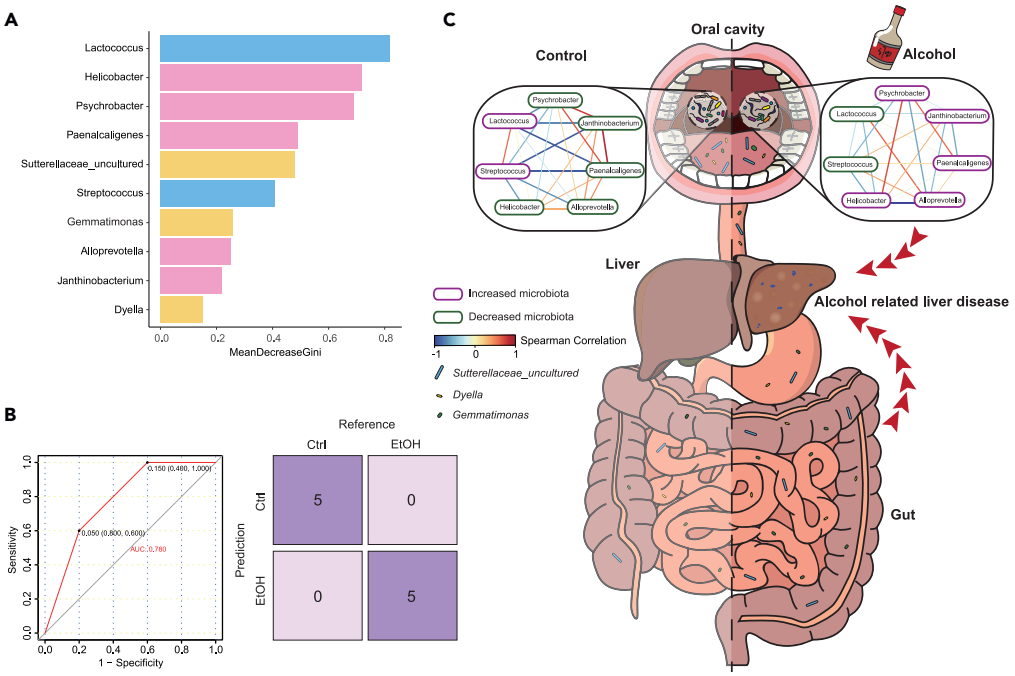

Figure 8. Alcohol drinking remodels oral microbiota to modulate ALD progression

(A) Bar plot of feature importance in the random forest model. Blue and pink bar: microbiota with decreased or increased abundance, respectively, in the oral samples collected in the evening following alcohol drinking. Yellow bar: bacteria which might shift along with the oral-gut axis to the intestine.

(B) Performance of the machine learning model discriminating mice fed with control or ethanol diet.

(C) The schematic diagram summarized our findings that alcohol feeding altered the bacterial diversities and compositions of oral microbiota in ethanol-fed mice as compared with control mice. The relationships among oral microbiota were also changed following alcohol feeding. Notably, the alterations of microbiota in the oral cavity were significantly correlated with ALD progression after ethanol intake. We further identified that three genera of bacteria, *Sutterellaceae_uncultured*, *Dyella*, and *Gemmatimonas*, might shift along the oral-gut axis to the intestine to promote the development of ALD.

The dysbiosis of oral microbiota could influence the systemic diseases, such as pancreatic cancer, liver cirrhosis, and cardiovascular disease.[10,13,18] Pathogenic bacteria and toxin metabolite in oral cavity may transfer to the systemic circulation via damaged oral mucosa.[24] Oral microbiota could also translocate to the gut and increase the permeability of the intestinal mucosa to invade to other tissues.[24] In this study, we observed that oral microbiota *Sutterellaceae_uncultured*, *Dyella*, and *Gemmatimonas* translocated to the intestine after alcohol feeding. One of the possibilities to explain the promotion of ALD progression by oral microbiota was that these oral microbiota or their metabolites might further shift from gut to liver, since evidence showed that the breach of the intestinal mucosal barrier by alcohol facilitated gut microbiota translocation to liver.[7,24] Nevertheless, all these findings indicated that the oral-gut axis might play an important role in the ALD progression.

Currently, liver biopsy is the golden standard of clinical diagnosis of ALD, which is invasive and has the risk of complications and sampling error.[25] Developing novel noninvasive diagnostic methods is thus urgently needed. Increasing evidence showed that oral microbiota could be biomarkers for the clinical diagnosis in several diseases, such as hepatocellular carcinoma[26] and colorectal carcinogenesis.[27] In this study, we established a machine learning model to predict ALD in mice; although the sample numbers were very limited, the model might still find its way into the prediction of ALD in clinical samples in the near future.

Collectively, our study demonstrated that the microbial structure and compositions as well as functions of oral microbiota could be regulated by alcohol drinking. We also discovered that the relationships among microbes in the oral cavity were reprogrammed with alcohol consumption. More importantly, the identified oral microbiota which translocated to the gut could be potential culprits for the progression of ALD. All these findings indicated that oral microbiota might be served as novel noninvasive biomarkers and therapeutic targets for ALD treatment.

Limitations of the study

The first limitation of the current study is that the findings observed in mice were not confirmed in the oral samples collected from the patients with ALD, so it is not sure whether the oral microbiota in humans has similar alteration following alcohol drinking, which deserves further investigation. The second limitation is that the mice numbers used to establish the machine learning model to predict ALD were very small, and the model was not tested in larger cohort, so the predictive power of this model also needs further verification in the future.

STAR★METHODS

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SUPPLEMENTAL INFORMATION

Supplemental information can be found online at https://doi.org/10.1016/j.isci.2023.107977.

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AUTHOR CONTRIBUTIONS

C.P. was responsible for experiments design, data acquisition and analysis, and manuscript writing; C.L. was responsible for data analysis; W.J. was responsible for performing experiments; D.Z. was responsible for data analysis in Figure 5; X.C. and X.Z. assisted in performing experiments; M.Y. assisted in data analysis in Figure 2D; L.W. was responsible for the study concept and design, study supervision, data analysis, and manuscript writing.

DECLARATION OF INTERESTS

The authors report no conflict of interest.

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STAR★METHODS

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Deposited data		
Raw data	This paper	SRA: PRJNA943134
Critical commercial assays		
ALT kit	Nanjing Jiancheng Bioengineering Institute	Cat#C009-2-1
AST kit	Nanjing Jiancheng Bioengineering Institute	Cat#C010-2-1
TIANamp Stool DNA Kit	TIANGEN	Cat#DP328
Control liquid diet	Bio-Serv	Cat#F1259SP
EtOH liquid diet	Bio-Serv	Cat#F1258SP
Experimental models: Organisms/strains		
C57BL/6J mice	Beijing Vital River Laboratory Animal Technology Company	N/A
Software and algorithms		
Prism 9	GraphPad	https://www.graphpad.com/
R package	R CRAN	https://www.r-project.org/
USEARCH v10.0.240	Edgar[28]	http://www.drive5.com/usearch
VSEARCH v2.13.6	Rognes et al.[29]	https://github.com/torognes/vsearch
Cytoscape v. 3.9.1.	Cytoscape Consortium	https://cytoscape.org/

RESOURCE AVAILABILITY

Lead contact

Further information and requests for any resources should be directed to and will be fulfilled by the lead contact, Lirui Wang (wanglirui@nju.edu.cn).

Materials availability

This study did not generate new unique reagents.

Data and code availability

- 16S rRNA sequencing data have been deposited at National Center for Biotechnology Information Sequence Read Archive database and are publicly available as of the date of publication. Accession numbers are listed in the key resources table.
- This article contains no original code.
- Any additional information required to reanalyze the data reported in this paper is available from the lead contact upon request.

EXPERIMENTAL MODEL AND STUDY PARTICIPANT DETAILS

Mice

Female C57BL/6J mice were obtained from Beijing Vital River Laboratory Animal Technology Company (Beijing, China). For ALD mice model, 10-12 week-old female mice were fed with control liquid diet for 5 days, followed by 10 days 6% EtOH liquid diet.[30] Mice from control group were fed with control liquid diet for the total of 15 days. At the 7:00-9:00 a.m. in the final day, mice were gavage with 31.5% ethanol (5 g/kg) or 45% maltose dextrin and sacrificed after 9 h.[30] Oral samples were collected every 7:00 a.m. in the morning and 4:00 p.m. in the evening. All animals were maintained under a specific pathogen-free (SPF) room with a constant temperature (25°C) and 12:12-h light/dark cycle. All animal procedures were approved by the institutional Animal Care and Use Committee of China Pharmaceutical University.

METHOD DETAILS

Plasma ALT and AST measurement

Blood from inferior vena cava was mixed with 0.5 M EDTA and centrifuged at 4°C, 10000 ×g for 5 min to collect plasma. ALT and AST levels were measured according to the manufacturer's protocols (Nanjing Jiancheng Bioengineering Institute).[31,32] Briefly, 20 μL of substrate

solution was mixed with 5 μL plasma and incubated at 37°C for 20 min. The mixture then added 20 μL of phenylhydrazine solution and incubated at 37°C for 30 min. Next, 200 μL 0.4 M NaOH was added to each well, and the absorbance at 510 nm was detect after 15 min standing at RT. For control wells, plasma was added after the first step of incubation, and the concentration of ALT and AST were calculated from standard curves.

Histology

The fixing and embedding of liver tissues and the H&E staining with sections were performed according to our previous studies.[31,33] Briefly, liver tissues were fixed with formalin, embedded in paraffin and sectioned in 5 μm. Liver sections were deparaffinized by xylene and concentration gradients of ethyl alcohol (100%, 95% and 70%). Hematoxylin and Eosin solution (Servicebio) were used for H&E staining. The score of steatosis showed the percentage of steatosis in liver sections with H&E staining.

Real-time PCR

The detailed procedure of real-time PCR was described in our previous studies.[31,33] Total RNA of liver tissues was extracted with RNAiso Plus (Takara), and then dissolved with DEPC-treated water (Sangon Biotech). HiScript III 1st Strand cDNA Synthesis Kit (Vazyme) was used to obtain cDNA. Real-time PCR was performed using ChamQ SYBR qPCR Master Mix (Vazyme) for 40 cycles of 10 s at 95°C and 30 s at 60°C on an ABI StepOnePlus instrument. Primer sequences for each gene were shown in the Table S1. Relative expressions of target genes were calculated using ΔΔCT and normalized to 18S as internal controls.

Genomic DNA extraction

The oral samples were collected using oral swabs by rubbing the oral cavity of mice for three times clockwise and scraping the inside and outside of the mice teeth. The oral swabs after sampling were rinsed in 500 μL Buffer SA (TIANamp Stool DNA Kit) for 50 times. The genomic DNA from oral samples and cecum samples were extracted by TIANamp Stool DNA Kit (DP328; TIANGEN). All the procedures were followed by the manufacturer's protocols.

16S rRNA sequencing and analysis

The isolated genomic DNA from mice oral cavity and cecum were used for 16S rRNA sequencing and analysis. The 16S ribosomal RNA (rRNA) V4 region was amplified and sequenced by the Illumina NovaSeq platform (Illumina, San Diego, CA) in Novogene Technology Co, Ltd (Beijing, China) to evaluate microbiota diversity. The analysis of raw sequencing data were described as before.[31,34] Briefly, the sequences were denoised in amplicon sequence variants (ASV) by UNOISE3 based on USEARCH[28] v10.0.240 (http://www.drive5.com/usearch) and VSEARCH[29] v2.13.6 (https://github.com/torognes/vsearch). The sequences were then aligned to SILVA138 database in order to calculate taxonomy table. α-diversity and β-diversity were calculated with USEARCH (https://drive5.com/usearch/manual/pipe_diversity.html) and online tools (https://www.microbiomeanalyst.ca/). The R package 'edgeR'[35] was utilized to find differential genus. Then LEfSe was performed online (http://huttenhower.sph.harvard.edu/galaxy) and the R package edgeR was obtained for identifying differential taxons.[35,36] The correlation analyses between microbiota and ALD phenotypes were performed using corr.test() function of psych package v.2.2.9 in R and visualized via corrplot and pheatmap package v.1.0.12. The significant correlations between oral microbiota and disease parameters were visualized by cytoscape v. 3.9.1. The raw data have been deposited in the National Center for Biotechnology Information Sequence Read Archive database (accession no. PRJNA943134).

Function prediction and data analysis

The ASV sequences analyzed by USEARCH using relevant procedures described in 16S rRNA sequencing and analysis, were further annotated with Greengenes database: https://greengenes.secondgenome.com. v.13_5.[37] Then the annotation results were adopted to predict metagenomic functions via PICRUST,[38] a suite implanted on a Galaxy website (http://huttenhower.sph.harvard.edu/galaxy/). KEGG pathways abundance of specific samples was extracted. R packages 'edgeR'[35] and 'limma'[39] were used to filter low-abundance features and find the differential ones with a threshold of adj.P.Val <0.05, which is a p value adjusted by Benjamini – Hochberg method.[40] After scaling by row, the scaled matrix was obtained for plotting heatmap via R package 'ComplexHeatmap' v. 2.15.1.[41,42] The visualization was used via ggplot2 v.3.4.1.

Establishment of machine learning model

The establishment of machine learning prospective model was described as before.[43] The machine learning model was built using 10 microbial features. The randomForest package in R was used for model construction. A 10-fold cross-validation was utilized to evaluate the model's performance. The pROC package was employed to generate the ROC curve, which assessed the discriminatory ability of this model.

QUANTIFICATION AND STATISTICAL ANALYSIS

Statistical analysis

In this study, data were presented as mean ± SEM and graphed using GraphPad PRISM 9. Two-tailed Student's *t* test and Mann-Whitney test were used for statistical analysis of the two-group comparisons; PerMANOVA test was used for β-diversity analysis. For the relative abundance of differential bacteria in control and ethanol group at the genus level, Wilcoxon rank sum tests and Paired samples t-test were utilized depending on whether the abundance of bacteria apply to normal distribution. *p value < 0.05, **p value < 0.01, ***p value < 0.001, ****p value < 0.001 were considered statistically significant.