

DR. JASMOHAN BAJAJ (Orcid ID : 0000-0003-4928-3681)

Article type : Original Articles

Editor : Janus Ong

Metabolomics and Microbial Composition Increase Insight into the Impact of Dietary Differences in Cirrhosis

I. Jane Cox^{1,2,*}, Ramazan Idiliman^{3*}, Andrew Fagan⁴, Dilara Turan³, Lola Ajayi^{1,2}, Adrien D. Le Guennec⁵, Simon D. Taylor-Robinson⁶, Fatih Karakaya³, Edith Gavis⁴, R. Andrew Atkinson⁵, Roger Williams^{1,2}, Masoumeh Sikaroodi⁷, Shahzor Nizam⁷, Patrick M. Gillevet⁷, Jasmohan S. Bajaj⁴.

¹Institute for Hepatology London, Foundation for Liver Research, London SE5 9NT, UK. ²Faculty of Life Sciences & Medicine, Kings College Hospital, London, UK. ³University of Ankara, Ankara, Turkey. ⁴Virginia Commonwealth University and McGuire Veterans Affairs Medical Center, Richmond, VA, USA. ⁵Randall Centre for Cell & Molecular Biophysics and Centre for Biomolecular Spectroscopy, King's College London. ⁶Department of Surgery and Cancer, Imperial College London, London, UK. ⁷George Mason University, Manassas, VA, USA.

*: Shared first authorship

Word count: 3804 [main body of manuscript]

Number of Figures: 4

Number of Tables: 2

This article has been accepted for publication and undergone full peer review but has not been through the copyediting, typesetting, pagination and proofreading process, which may lead to differences between this version and the [Version of Record](#). Please cite this article as [doi: 10.1111/LIV.14256](https://doi.org/10.1111/LIV.14256)

This article is protected by copyright. All rights reserved

List of Abbreviations:

HBV: hepatitis B virus

HCV: hepatitis C virus

HE: hepatic encephalopathy

LEFSe: linear discriminant function effect size

MaAsLin: Multivariate Association with Linear Models

MELD: Model for End-Stage Liver Disease

NASH: non-alcoholic steatohepatitis

NMR: nuclear magnetic resonance

NSBB: nonselective beta-blockers

OPLS-DA: orthogonal partial least squares discriminant analysis

PiCRUST: Phylogenetic Investigation of Communities by Reconstruction of Unobserved States

PLS-DA: partial least squares-discriminant analysis

SVR: sustained virological response

Conflict of Interest: The authors have declared that no conflict of interest exists

Statement of financial support: This work was partly supported by grants from VA Merit Review I0CX001076, R21TR002024 from NCATS to JSB and a grant from the Turkish Association for the Study of the Liver to RI. SDT-R is grateful to the United Kingdom National Institute for Health Research Biomedical Facility at Imperial College London for infrastructure support. The NMR Facility of the Centre for Biomolecular Spectroscopy was funded by British Heart Foundation, Wellcome Trust and King's College London.

Trial registration: Not applicable; this is an observational cohort study

Corresponding Author: Jasmohan S Bajaj, MD, Division of Gastroenterology, Hepatology and Nutrition, Virginian Commonwealth University and McGuire VA Medical Center, 1201 Broad Rock Boulevard, Richmond, VA 23221, USA. Telephone: (804) 675 5802. Fax: (804) 675 5816. Email: jasmohan.bajaj@vcuhealth.org

Abstract

Background & Aims: Dietary changes can modulate gut microbiota and interact with cirrhosis. Our prior study demonstrated that microbial diversity was higher in Turkish versus USA cirrhotics,

which was associated with lower risk of 90-day hospitalizations. We aimed to define gut microbial functional and metabolomic changes to increase insight into benefits of the Mediterranean compared to Western diets.

Methods: 139 Turkish (46 controls/50 compensated/43 decompensated) and 157 American subjects (48 controls/59 compensated/50 decompensated) were studied. Turkish subjects consumed a modified Mediterranean diet with daily fermented milk intake while Americans consumed a Western diet. Predicted gut microbial functionalities and plasma metabolomics were compared between/within countries. Correlation network differences between microbiota and metabolites in cirrhotics from Turkey versus USA were evaluated.

Results: Predicted microbial function showed lower amino acid, bioenergetics and lipid pathways, with functions related to vitamin B, glycan, xenobiotic metabolism, DNA/RNA synthesis, in Turkey compared to USA cirrhotics. Plasma metabolomics demonstrated higher relative lactate levels in Turkey versus USA. The metabolite changes in decompensated cirrhosis, compared to controls, showed similar trends in Turkey and USA, with reduced lipids and phosphocholines. Phosphocholines were significantly lower in patients hospitalized in 90 days ($p=0.03$). Correlation networks in cirrhotics demonstrated linkage differences between beneficial taxa, *Blautia* and *Oscillispira*, and lactate and unsaturated lipids, in Turkey compared to American patients.

Conclusions: A modified Mediterranean diet was associated with altered plasma metabolomics and beneficially alters microbiota functionality and correlations compared to Western diet in cirrhosis. These altered diet-microbial interactions could potentially affect the 90-day hospitalization risk. [Abstract word count: 250]

Key words: cirrhosis, Mediterranean diet, Western diet, metabolomics, gut microbial function, fermented milk products.

Lay summary: We show that the different diets followed in Turkey and America contribute to alterations in gut microbial function and plasma metabolomics observed in patients with cirrhosis, which may explain the lower 90-hospitalization in Turkish patients. This should help in the design of dietary interventions across international cohorts of patients with cirrhosis.

INTRODUCTION

Patients with cirrhosis have an altered gut-liver axis, which can influence clinical and psychosocial outcomes¹. Differences in microbial composition are important as potential biomarkers of disease progression in cirrhosis¹. Indeed, prior studies have shown an increased relative abundance of gram-negative Proteobacteria and lowering of gram-positive Firmicutes as the disease progresses². Changes in gut microbial composition and function are a major determinant of the gut-liver axis and differences in cultural, ethnic and dietary practices can modulate the gut microbiota composition even within associated disease³⁻⁵. These changes are affected by macronutrient and fermented food intake, which can drive beneficial changes in gut microbiota for those on whose diet typically involves more fermented, probiotic foods such as a Mediterranean diet, compared to typical Western diets in patients with cirrhosis⁶. Our previous results, comparing cirrhotics from USA and Turkey, demonstrated lower Shannon diversity in all American subjects compared to their Turkish counterparts, which reflects a poor dietary intake of probiotic constituents such as fermented milk products, and was associated with lower risk of 90-day hospitalizations⁶. This study showed that despite having compensated and decompensated cirrhosis, the Shannon diversity of Turkish patients was similar to their respective controls.

However, the role of gut microbial function and its impact on the differences between cirrhotic patients on a modified Mediterranean, compared to Western diets, is unclear. Therefore, the focus of this study was the functional analysis of the microbiota in cirrhosis, determined in the same Turkey-USA control/cirrhotic cohort⁶, using the bioinformatics tool, (PiCRUST: Phylogenetic Investigation of Communities by Reconstruction of Unobserved States⁷) and metabolomics analyses. Metabolomics can be used to analyze the systemic impact of dietary factors on gut microbiota function from bio-fluids using either mass spectrometry or nuclear magnetic resonance (NMR) spectroscopy techniques^{8,9}. Certain metabolites may be linked with a specific species or group of gut bacteria¹⁰. However, other metabolites, for example lactate and 3-hydroxybutyrate, may be related to a diet rich in acidified foods⁸ and can reflect interactions between disease states, diet and microbial function.

Our aim was to define predicted gut microbial functional changes and plasma NMR metabolomics associated with Western and modified Mediterranean diets in healthy and cirrhotic subjects from USA and Turkey to assess beneficial effects of either diet.

METHODS

Three groups of subjects, healthy controls, compensated cirrhotic and decompensated cirrhotic outpatients were recruited from centers in USA (Virginia Commonwealth University and McGuire

VA Medical Center, Richmond, Virginia) and Turkey (Ankara University School of Medicine, Ankara, Turkey).

The diagnosis of cirrhosis was based on liver biopsy if available, ultrasound-based elastography or clinical, biochemical and radiological evidence of cirrhosis. Decompensation was defined as variceal bleeding in the immediate past, ascites under treatment, hepatic encephalopathy under treatment and those with jaundice¹¹. Patients with alcohol misuse and/or illicit drug usage (defined by DSM-V criteria)¹², inability to provide samples and those who were hospitalized within the last 30 days were excluded from the study. The healthy controls were free of chronic diseases, were not on any prescription medications and had normal liver function.

The subjects underwent a dietary evaluation over the previous week which inquired about their overall dietary preferences (vegan, vegetarian, or predominantly non-vegetarian) using a specifically designed food-frequency questionnaire. Specifically, we inquired about coffee, tea, caffeinated/decaffeinated carbonated drinks, fermented foods (yogurt, ayran/kefir, curds), other dairy products (milk, cheese), eggs, cereals and grains (breakfast cereals, rice, white bread and wheat bread), meat intake (lamb/mutton, pork, beef, fish, poultry, other seafood) and chocolate. Coding was performed according to serving size (Supporting Table 1), based on daily or >1/week frequencies, depending on the food item. In the decompensated group, prior clinical events included variceal bleeding, HE, jaundice and ascites, as well as the presence of varices had been recorded¹³. Laboratory values (complete blood count, MELD score, serum albumin, serum sodium) at the time of sample collection were recorded. In addition, current medications [lactulose, rifaximin, fiber supplements, proton pump inhibitors (PPI), non-selective beta-blockers (NSBB), antiviral therapy for hepatitis B], as well as lifestyle issues (smoking and alcohol history) were obtained at the same time¹⁴. Co-morbid conditions such as diabetes and metabolic syndrome and history of sustained virological response (SVR) from hepatitis C were also assessed^{15,16}.

Predicted functionality changes of the microbiota

All patients underwent stool collection for microbiota analysis. Microbial DNA were isolated from stool samples and analysed using 16srRNA analysis as previously described and reported¹⁷. We performed PiCRUST⁷ to determine predicted functionality differences between sites. We compared PiCRUST data using Linear discriminant analysis effect size (LEFSe) for the entire group for compensated, decompensated and control subjects and between cirrhotic patients within and between sites¹⁸.

Metabolomic analysis

Plasma collected in a fasted state from all individuals was analysed using NMR spectroscopy to determine metabolomics using lipidomic and small metabolite platforms. Signal intensities of

metabolite regions were obtained using intellibucketing within BioRad KnowItAll® Informatics, Metabolomics Edition v17.0. Principal component analysis (PCA) between six groups (USA and Turkey: control, compensated and decompensated) was performed after the data were mean-centred and normalised. The binned NMR spectral data was uploaded into MetaboAnalyst v4.0 program¹⁹ for partial least squares-discriminant analysis (PLS-DA), where $Q^2 > 0.40$ indicates good separation, and orthogonal partial least squares discriminant analysis (OPLS-DA). The variable importance in projection (VIP) scores allowed an assessment of the contribution of each metabolite region to the PLS-DA model. We also performed these analyses between patients with and without specific medication when differences were found between groups. Signal levels of selected metabolites, relative to total signal intensity, were compared using post-hoc ANOVA and t-tests with Benjamini-Hochberg correction for multiple comparisons. Further details are in supporting data. We also compared the metabolites that emerged as different between patients with cirrhosis who got hospitalized in 90 days versus those who remained free of hospitalization using Mann-Whitney tests. In addition, individual metabolites for controls underwent stepwise linear regression using age, gender, USA/Turkey as independent variables. Individual metabolites in cirrhotic patients were analysed using stepwise linear regression with age, gender, compensated/decompensated and USA/Turkey as independent variables.

Correlation networks

Plasma metabolomics were linked with microbial taxa at the genus level using an R program and visualized in Cytoscape. Correlations between microbiota and metabolites that were different within USA and Turkey at $p < 0.001$ significance level were analysed. Dietary components were not added to this given that most subjects within the country-specific cohorts consumed a similar diet. Therefore, dividing the population as USA vs Turkey was enough to represent the underlying dietary changes.

Multi-variable analyses:

MaAsLin (Multivariate Association with Linear Models), which studies associations between clinical metadata and microbial community abundance²⁰, was performed for the controls and the cirrhosis group separately. For patients with cirrhosis, MaAsLin inputs included demographics, cirrhosis severity details (MELD score, prior complications, medications, alcoholic etiology) and changes between USA and Turkey, while for controls it only included demographics. A p-value < 0.05 with a false discovery rate q-value < 0.05 was considered significant.

As published before⁶, we performed multi-variable binary logistic regression analyses for all-cause hospitalization, the variables were divided into Clinical (Age, Male Gender, MELD, Smoking, Diabetes, Albumin, Na, Prior Ascites, Prior HE, Prior Variceal Bleed, Prior Jaundice, Alcohol etiology and Presence of Varices), medicines (Lactulose, Rifaximin, PPI, Beta Blockers),

diet (Coffee/Tea, Yogurt, Milk, Fruits, Vegetables, Eggs, Cheese, Rice, Cereals, Bread-White, Bread-Whole Wheat, Bread-Other, Lamb, Pork, Beef, Fish, Other Seafood, Poultry, Caffeinated Carbonated Drinks, Decaffeinated Carbonated Drinks, Alcohol and Chocolate) and Shannon diversity. Due to this large number of variables, separately, for each domain, a backwards elimination procedure was performed with the logistic regression model where any variable significant at the $p < 0.20$ significance level was retained. Ultimately 90-day hospitalization was used as the dependent variable.

Study approval

Written informed consent was obtained from all subjects and the protocols were approved by the local IRBs of each institution.

RESULTS

Cohort characteristics

Subjects: The etiology of cirrhosis was predominantly hepatitis C and alcohol in USA, while it was more related to hepatitis B (HBV), hepatitis C (HCV) and non-alcoholic steatohepatitis (NASH) in Turkey (Table 1). The US and Turkish cirrhotic population were statistically similar in age, MELD score, PPI use, metabolic syndrome, and proportion with decompensated cirrhosis (Table 1). The Turkish controls were significantly younger than all other groups. The American cirrhotic cohort had a higher proportion of men, those with recent alcohol use, lower serum sodium and platelet counts, NSBB use and prior variceal bleeding, and those with prior HE and those on lactulose and rifaximin. There was a higher proportion with prior jaundice in the Turkish cohort. SVR rates, prior ascites and other blood indices were similar between groups. Over 90 days, 12 patients in the Turkish and 29 in the USA cohort ($p=0.016$) were hospitalized as previously reported, but given the overall low numbers, we divided the groups into hospitalized vs not-hospitalized for the entire cohort⁶. The mean time between enrolment and hospitalization was 46.8 ± 31.9 days. The major causes of hospitalization were acute kidney injury/ascites ($n=13$), infections ($n=7$), hepatic encephalopathy ($n=6$), HCC-related ($n=4$), variceal bleeding ($n=3$) and liver-unrelated in the rest. Patients who were ultimately hospitalized demonstrated a lower Shannon diversity index (6.6 ± 1.8 vs 7.3 ± 1.3 , $p=0.02$). None of these patients received a liver transplant or died prior to the hospitalization, which is why we only used hospitalization as the end-point.

Dietary details: All subjects were non-vegetarian and had similar caloric intake and macronutrient proportions (Table 1). Western diet was the norm in the US population with relatively low consumption of fermented foods and high coffee, pork and fish consumption

(Supporting Table 2). In patients with cirrhosis, US patients consumed more carbonated drinks compared to Turkish patients. The Turkish cirrhotic cohort consumed a diet approximating to a modified Mediterranean diet, which was rich in fermented foods such as yogurt and vegetables. In addition, they demonstrated a higher tea consumption, as well as avoidance of pork and alcohol. In controls recruited from Turkey, there was a greater coffee and carbonated drink consumption and lower tea and fermented milk product consumption than either of the Turkish cirrhotic patient groups.

Predicted functionality changes of the microbiota

Comparison of subject groupings between countries (Figure 1): There were major differences in PiCRUST between controls from USA versus controls from Turkey (Figure 1A). Microbiota in the controls from the USA had a greater metabolism related to amino acid, bioenergetics and lipid pathways while Turkish controls had microbiota that were more likely to express functions related to vitamin B, glycan, xenobiotic metabolism, and DNA/RNA synthesis. The USA compensated patients had higher predicted microbial functionality, related to bioenergetics, bacterial toxin production and amino acid metabolism and lower bile, peptidoglycan, and RNA/DNA synthesis, compared to Turkish compensated patients (Figure 1B). Differences were even greater when decompensated patients were compared across countries. Again, the USA decompensated patients had greater predicted microbial functionality related to bioenergetics, amino acids, bacterial motility and assembly with xenobiotic handling and lower RNA/DNA and peptidoglycan metabolism, compared to their Turkish counterparts (Figure 1C).

Controls vs cirrhosis (Supporting Figure 1): There were significant differences in the predicted functionality of microbiota for the control group compared to each of the compensated and decompensated cirrhosis groups, with the populations combined (Supporting Figure 1A and 1B). In controls compared to compensated cirrhosis, there was higher amino acid, glycan and lipid metabolic pathway expression, while environmental processing systems were overexpressed in compensated cirrhosis. Similarly, amino acid, glycan and lipid pathways were more expressed and environmental pathways and purine metabolism were less expressed in controls compared to decompensated cirrhotic patients.

Compensated vs decompensated cirrhosis (Figure 2): In the USA, decompensated cirrhotic patients had greater vitamin B6, neomycin and drug metabolism and lower cytochrome p450 and nucleotide metabolism compared to compensated patients (Figure 2A). In the Turkish cohort, there was again a higher nucleotide metabolism along with amino acid metabolism with lower predicted microbial functionality related to bacterial motility/chemotaxis and intracellular processes in decompensated patients (Figure 2B).

Metabolomics

Comparison of subject groupings between countries (Figure 3A): There were differences between controls and decompensated patients from USA and Turkey (PLS-DA Q2 0.60, 0.45 respectively), although less pronounced differences between compensated patient groups (PLS-DA Q2 0.29), mainly due to a higher lactate levels in each Turkey sub-groups compared to the USA groupings. OPLS-DA plots between corresponding cohorts between countries are shown in Figure 3A and representative NMR spectra in Supporting Figure 2.

We also compared those taking, or not taking, rifaximin or lactulose for patients with decompensated cirrhosis between countries (Supporting Figure 3). The results demonstrated that, regardless of rifaximin and lactulose use, the major comparison between patients with decompensated cirrhosis not taking rifaximin was similar to the overall comparison between decompensated cirrhosis patients between USA and Turkey (Figure 3A). Therefore, this suggests taking rifaximin or lactulose was not the main factor contributing to the metabolomic differences seen between patients with decompensated cirrhosis from USA vs Turkey (Supporting Figure 3)

Comparison of controls and cirrhosis within countries (Supporting Figure 4): Within the USA group, the difference in plasma metabolites was most pronounced between controls and decompensated patients (PLS-DA Q2 0.59), rather than controls and compensated patients (PLS-DA Q2 0.17). There were differences in plasma metabolites within the Turkish cohort, despite similar microbial diversity, which was marked between controls and compensated cirrhosis but greatest between controls vs decompensated cirrhosis (PLS-DA Q2 control-compensated 0.40, PLS-DA Q2 control-decompensated 0.69).

Cirrhosis comparison within countries (Figure 3B): There was some overlap between compensated and decompensated cirrhotic patients within both the USA and Turkish cohorts (PLS-DA Q2=0.28 and 0.28 respectively).

Individual metabolites driving the differences between subject groupings (Table 2 and Supporting Table 3):

The Turkish control group had higher relative lactate levels but lower glucose levels compared to the USA controls, with a trend for lower lipid-CH₂ and lipid-CH₃ (VLDL) levels. Lactate levels were also higher in decompensated patients from Turkey compared to the USA. Within the USA group, relative lipid-CH₂, lipid-CH₃ and phosphocholines were higher in controls compared to decompensated cirrhosis; and relative glucose levels were lowest in controls. Within the Turkish cohort, relative levels of lipid-CH₂ and phosphocholines were higher in controls compared to cirrhotics and in compensated compared to decompensated cirrhotics; relative lactate levels were higher in controls compared to cirrhotic patients; relative glucose levels were higher in both

cirrhosis groups compared to controls. Representative NMR spectra are shown in Supporting Figure 3.

When regressions were performed for individual metabolites using age, gender and USA/Turkey in the control population, lipid-CH₂, lipid -CH=CH-CH₂-CH=CH- (polyunsaturated lipid, L6) and lactate were significantly differentially predicted by USA vs Turkey (p<0.001) in addition to the age variables. In the cirrhotic group, lipid-CH₂, L6, lactate, acetate, and phosphocholine were significantly predicted by USA vs Turkey (p<0.001) while compensated/decompensated status was additionally associated with formate, lactate, L6, GPC, choline, alanine and phosphocholine (all p<0.01).

Comparison of metabolites in patients with and without hospitalizations: We found significantly higher phosphocholine in the patients who were not hospitalized [median (IQR): hospitalized 0.014(0.05) vs not hospitalized 0.011(0.06), p=0.03]. Neither lactate, lipid-CH₂ and lipid-CH₃ were significantly different between those who experienced hospitalization at 90 days compared to the rest.

Correlation Network differences (Figure 4/Supporting Figure 5):

We found several changes between the relationship of plasma metabolites and gut microbiota in Turkish compared to US compensated and decompensated cirrhotic patients (Supporting Figure 5). Specifically, we created sub-networks based on specific microbial genera (Figure 4A-D). Oscillibacter, and SCFA-producing genus, had relationships with constituents of Enterobacteriaceae that were more negative in the American cohorts (Figure 4A). The linkage between Oscillibacter and genera with Lachnospiraceae were positive in the Turkish, but negative in the US patients (Figure 4C). Blautia, another SCFA-producing genus, was negatively associated with relative plasma lipid -CH=CH-CH₂-CH=CH- levels (polyunsaturated lipid, L6) in Turkey, but not in US-based cohorts (Figure 4D). Interactions between Lachnospiraceae components were positive in both cohorts (Figure 4D). Linkages between Lachnospiraceae taxa and Bacteroides and Alistipes were positive in Turkey and turned negative in USA. Using Bacteroides as the sub-network (Figure 4B), Clostridium taxa were more positive in USA but genera belonging to Lachnospiraceae, Escherichia/Shigella and Colinsella were negative in USA and positive in Turkey. When Escherichia/Shigella were used the focus, Ruminococaceae components were even more negative in USA compared to Turkey, while lactate was negatively correlated with Escherichia and Shigella in USA without any relationship in the Turkish cohort. Relative plasma formate was negatively linked with Parasutterella in Turkey, which became positive in USA cohort (Supporting Figure 5).

Multi-variable analyses:

Logistic regression: The variables that were retained for each domain (Clinical: MELD, Prior Ascites; Treatment: lactulose and Non-selective Beta Blockers; Diet: Vegetables, Cereals and Coffee/tea) were then placed into a model simultaneously and another backwards elimination logistic regression model was performed, this time retaining variables significant at the $p < 0.05$ significance level. The variables significant at this stage were MELD, cereals, vegetables, coffee/tea and prior ascites. When Shannon index was introduced, the significant model consisted of diversity (OR 0.78, CI 0.59-0.96, $p=0.04$), cereals (0.19, CI 0.05-0.77, $p=0.02$), vegetables (0.17, CI 0.05-0.65, $p=0.008$), coffee/tea (0.38, CI:0.16-0.91, $p=0.03$) and prior ascites (4.72, CI 2.08-10.73, $p<0.001$).

MaAsLin outputs for the patients with cirrhosis demonstrated changes in species level based on (a) MELD score (b) age and (c) USA vs Turkey. Species associated with age belonged to Lachnspiraceae (*Clostridium symbiosium* and *Dorea formicigenerans*) with *Shigella Boydii* (Supporting table 4). The MELD score was associated with *Enterobacteriaceae_Klebseilla* (p value $5.12E-05$ and q value 0.02). For USA vs Turkey comparisons, the two species that were important were *Veillonellaceae_Dialister* (p value $9.55E-06$, q value 0.003), and *Prevotella_copri* (p value $5.48E-07$, q value 0.0009). None of the other variables, including gender, alcohol etiology, individual medication use, history of cirrhosis complications or diabetes showed multi-variable associations with the microbiota.

MaAsLin results between controls did not show any microbiota that were different between groups that were specifically associated with age, gender or ethnicity.

DISCUSSION

In this study we found differences in predicted gut microbiota functionality, plasma metabolomic profiles and correlation networks between and within Turkish and USA cirrhotic cohorts.

Differences in gut microbiota functionality and plasma metabolites were also observed between Turkish and USA control groups, which could factor into the overall benefit of the Mediterranean diet in cirrhosis in the Turkish cohort.

Alterations in gut microbial composition and function can be affected by multiple factors, including diseases, ethnicity, and diet²¹. Performing a comparison between countries, the Turkish controls showed lower predicted microbial functionality related to amino acid, bioenergetics and lipid metabolism but were more likely to express functions related to vitamin B and glycan metabolism. Relative plasma lactate levels were greater in the Turkish control group, compared to the USA cohort, reflecting increases in consumption of lactate present in fermented dairy products in the

diet^{8,22}. On the other hand, in the USA control group, relative lipid-CH₂ and lipid-CH₃ (VLDL) levels tended to be higher when compared to the Turkish control group, even though both diets had a similar total calorie proportions from fat. As the USA control group had a richer abundance of bacterial functions relating to lipid metabolism, this suggests a different mechanism for lipid nutrient metabolism compared to the Turkish counterparts, which is likely related to the Western diet^{23,24}.

These changes were further illustrated by the higher energy harvesting (bioenergetics, amino acid production) and lower bile acid metabolism, peptidoglycan and RNA/DNA synthesis in USA cirrhotic compared to Turkish compensated and decompensated patients. In addition, USA groups showed higher potentially harmful bacterial functionality related to toxin production, chemotaxis/motility and xenobiotic handling²⁵. These findings in the Turkish cohort suggest greater bacterial metabolism of bile acids likely related to more gram positive rather than gram-negative taxa due to the peptidoglycan cell wall and greater bacterial replication, which are associated with an optimal intestinal milieu²⁶. When patients with decompensated cirrhosis were compared, we found significant plasma metabolite differences with higher lactate and lipid-CH₂ levels in decompensation in Turkey. Since the Turkish cirrhotic population had a lower 90-day hospitalization, these microbial functional changes and plasma metabolomic features could likely contribute towards this benefit²⁷.

This study on predicted microbiota functionality and plasma metabolomics demonstrated systematic differences related to dietary changes in two distinct populations that were variably modulated by the cirrhotic state. Changes between microbial-metabolite linkages were observed in cirrhosis with genera associated with benefit (*Oscillibacter*, *Blautia*) and those associated with negative consequences such as *Escherichia/Shigella* and *Bacteroides* that differed between USA and Turkey. *Blautia* are significant acetate producers, which are more common in the presence of a fermented milk-containing and fiber-rich diets²⁸, and acetate is the principal gut microbial-based stimulus for beta-oxidation of fatty acids. Thus, in the Turkish diet, the acetate produced by *Blautia* may be the stimulus for burning fat, which would be consistent with the negative correlation noted between L6 and *Blautia*. On the other hand, a positive correlation was noted between L6 and *Blautia* in the USA cohort. Other species belonging to Prevotellaceae and Veillonellaceae were also different between USA and Turkey on multi-variable modeling in the cirrhosis group. These are associated with greater “oralization”, worsening intestinal inflammation and poor outcomes^{29,30}. The effect of diet and microbiota was also confirmed on multi-variable logistic regression, where a higher microbial diversity, along with cereals, vegetables and coffee/tea intake was associated with lower hospitalization independent of cirrhosis severity,

demographics and concomitant medication use. These altered diet-microbial interactions could contribute towards the lower 90-day hospitalizations previously observed in those with higher microbial diversity²⁷.

Despite the between country changes above, there were also patterns by which cirrhotic patients differed from controls regardless of USA or Turkey. In both the American and Turkish cohorts, reduced lipid-CH₂ and phosphocholine were observed in decompensated patients compared to controls. Phosphocholines, associated with cell and hepatic regeneration, were more abundant in controls and compensated patients compared to cirrhotic and decompensated patients respectively³¹. Higher phosphocholines were also protective against 90-day hospitalizations in the entire cohort extending a study of survival in decompensated cirrhotic patients into an outpatient compensated/decompensated group³². While the mechanisms are unclear, these could be linked with *in vivo* hepatic MR spectroscopy findings that reflect hepatic accumulation of both phosphoethanolamines and phosphocholines and be an index of hepatocyte regeneration³³.

Higher levels of lipid-CH₃ (LDL) were also observed in relatively healthier groups within and between countries. Synthesis of LDL is a key hepatic function affected by the diet³⁴. However, further studies are required to define a causal relationship between dyslipidemia and apoptosis/necrosis at the hepatic level²⁵. This pattern also demonstrates that there do remain some underlying metabolic abnormalities related to cirrhosis that are stable across countries, remained significant on multi-variable analysis and therefore are unlikely to be related to diet or microbiota differences.

The study may be limited overall by differences in disease etiology and medication use between cirrhosis cohorts and possibly by the younger age of Turkish controls. However, comparison between subgroups of decompensated cirrhosis on or not on rifaximin or lactulose and the multi-variable analyses did not change the overall differences. However, between USA and Turkish cohorts, measures such as the MELD score, metabolic syndrome, diabetes and BMI were statistically similar between groups. We performed multi-variable regression to further confirm these changes. We only focused on hospitalizations given the relatively small number of patients and further studies to evaluate liver transplant and risk of death are required. Given the uniform dietary pattern within a population, we used a categorization of yes/no in the dietary evaluation, rather than a quantitative measure, and therefore this was not specifically added to the correlation network. There could be factors other than diet, including environmental that could impact these correlations and the results need to be interpreted in that light.

We conclude that Mediterranean and Western diets contribute to alterations in microbial function and metabolomics in patients with cirrhosis that were linked with beneficial microbiota in subjects on the Mediterranean diets. These differential effects could contribute to the relatively better prognosis of patients on a Mediterranean diet and should increase insight into designing dietary interventions across international cohorts of patients with cirrhosis.

References

1. Acharya C, Bajaj JS. The Microbiome in Cirrhosis and its Complications. *Clin Gastroenterol Hepatol*. 2018.
2. Chen Y, Yang F, Lu H, et al. Characterization of fecal microbial communities in patients with liver cirrhosis. *Hepatology*. 2011;54(2):562-572.
3. Rothschild D, Weissbrod O, Barkan E, et al. Environment dominates over host genetics in shaping human gut microbiota. *Nature*. 2018;555(7695):210-215.
4. Wu GD, Chen J, Hoffmann C, et al. Linking long-term dietary patterns with gut microbial enterotypes. *Science*. 2011;334(6052):105-108.
5. Zmora N, Suez J, Elinav E. You are what you eat: diet, health and the gut microbiota. *Nat Rev Gastroenterol Hepatol*. 2019;16(1):35-56.
6. Bajaj JS, Idilman R, Mabudian L, et al. Diet affects gut microbiota and modulates hospitalization risk differentially in an international cirrhosis cohort. *Hepatology*. 2018.
7. Langille MG, Zaneveld J, Caporaso JG, et al. Predictive functional profiling of microbial communities using 16S rRNA marker gene sequences. *Nature biotechnology*. 2013;31(9):814-821.
8. Pedersen SM, Nielsen NC, Andersen HJ, et al. The serum metabolite response to diet intervention with probiotic acidified milk in irritable bowel syndrome patients is indistinguishable from that of non-probiotic acidified milk by 1H NMR-based metabonomic analysis. *Nutrients*. 2010;2(11):1141-1155.
9. Grimaldi R, Gibson GR, Vulevic J, et al. A prebiotic intervention study in children with autism spectrum disorders (ASDs). *Microbiome*. 2018;6(1):133.
10. Li M, Wang B, Zhang M, et al. Symbiotic gut microbes modulate human metabolic phenotypes. *Proc Natl Acad Sci U S A*. 2008;105(6):2117-2122.
11. Schuppan D, Afdhal NH. Liver cirrhosis. *Lancet*. 2008;371(9615):838-851.
12. Wishart DS, Jewison T, Guo AC, et al. HMDB 3.0--The Human Metabolome Database in 2013. *Nucleic Acids Res*. 2013;41(Database issue):D801-807.
13. Albilllos A, Garcia-Tsao G. Classification of cirrhosis: the clinical use of HVPG measurements. *Dis Markers*. 2011;31(3):121-128.
14. Merli M, Lucidi C, Di Gregorio V, et al. The chronic use of beta-blockers and proton pump inhibitors may affect the rate of bacterial infections in cirrhosis. *Liver Int*. 2015;35(2):362-369.
15. Forslund K, Hildebrand F, Nielsen T, et al. Disentangling type 2 diabetes and metformin treatment signatures in the human gut microbiota. *Nature*. 2015;528(7581):262-266.

16. Heidrich B, Vital M, Plumeier I, et al. Intestinal microbiota in patients with chronic hepatitis C with and without cirrhosis compared with healthy controls. *Liver Int.* 2017.
17. Gillevet P, Sikaroodi M, Keshavarzian A, Mutlu EA. Quantitative assessment of the human gut microbiome using multitag pyrosequencing. *Chem Biodivers.* 2010;7(5):1065-1075.
18. Segata N, Izard J, Waldron L, et al. Metagenomic biomarker discovery and explanation. *Genome biology.* 2011;12(6):R60.
19. Chong J, Soufan O, Li C, et al. MetaboAnalyst 4.0: towards more transparent and integrative metabolomics analysis. *Nucleic Acids Res.* 2018;46(W1):W486-W494.
20. Morgan XC, Tickle TL, Sokol H, et al. Dysfunction of the intestinal microbiome in inflammatory bowel disease and treatment. *Genome biology.* 2012;13(9):R79.
21. Allaband C, McDonald D, Vazquez-Baeza Y, et al. Microbiome 101: Studying, Analyzing, and Interpreting Gut Microbiome Data for Clinicians. *Clin Gastroenterol Hepatol.* 2019;17(2):218-230.
22. de Vrese M, Barth CA. Postprandial plasma D-lactate concentrations after yogurt ingestion. *Z Ernahrungswiss.* 1991;30(2):131-137.
23. Park Y, Kim SB, Wang B, et al. Individual variation in macronutrient regulation measured by proton magnetic resonance spectroscopy of human plasma. *Am J Physiol Regul Integr Comp Physiol.* 2009;297(1):R202-209.
24. Kindt A, Liebisch G, Clavel T, et al. The gut microbiota promotes hepatic fatty acid desaturation and elongation in mice. *Nat Commun.* 2018;9(1):3760.
25. Shawcross DL. Is it time to target gut dysbiosis and immune dysfunction in the therapy of hepatic encephalopathy? *Expert Rev Gastroenterol Hepatol.* 2015;9(5):539-542.
26. Kakiyama G, Pandak WM, Gillevet PM, et al. Modulation of the fecal bile acid profile by gut microbiota in cirrhosis. *J Hepatol.* 2013;58(5):949-955.
27. Bajaj JS, Idilman R, Mabudian L, et al. Diet affects gut microbiota and modulates hospitalization risk differentially in an international cirrhosis cohort. *Hepatology.* 2018;68(1):234-247.
28. Rios-Covian D, Ruas-Madiedo P, Margolles A, Gueimonde M, de Los Reyes-Gavilan CG, Salazar N. Intestinal Short Chain Fatty Acids and their Link with Diet and Human Health. *Front Microbiol.* 2016;7:185.
29. Scher JU, Sczesnak A, Longman RS, et al. Expansion of intestinal *Prevotella copri* correlates with enhanced susceptibility to arthritis. *Elife.* 2013;2:e01202.
30. Bajaj JS, Acharya C, Fagan A, et al. Proton Pump Inhibitor Initiation and Withdrawal affects Gut Microbiota and Readmission Risk in Cirrhosis. *Am J Gastroenterol.* 2018.

- Accepted Article
31. Li Z, Agellon LB, Allen TM, et al. The ratio of phosphatidylcholine to phosphatidylethanolamine influences membrane integrity and steatohepatitis. *Cell Metab.* 2006;3(5):321-331.
 32. McPhail MJ, Shawcross DL, Lewis MR, et al. Multivariate metabotyping of plasma predicts survival in patients with decompensated cirrhosis. *J Hepatol.* 2016;64(5):1058-1067.
 33. Menon DK, Sargentoni J, Taylor-Robinson SD, et al. Effect of functional grade and etiology on in vivo hepatic phosphorus-31 magnetic resonance spectroscopy in cirrhosis: biochemical basis of spectral appearances. *Hepatology.* 1995;21(2):417-427.
 34. McIntyre N. Plasma lipids and lipoproteins in liver disease. *Gut.* 1978;19(6):526-530.

Table 1: Characteristics of the cohort

	USA (n=157)				Turkey (n=139)			
	All	Control (n=48)	Comp (n=59)	Decomp (n=50)	All	Control (n=46)	Comp (n=50)	Decomp (n=43)
Age†		62.1±13.6	61.0±7.9	61.0±6.8		42.4±8.0	59.9±10.2	60.4±12.2*
Males†	121	27	48	46*	79	23	29	27
BMI		25.6±5.8	26.3±7.4	27.9±11.9		26.2±6.0	27.0±9.2	29.0±6.1
Diabetes	29	-	14	15	28	-	17	11
Metabolic syndrome	35	-	16	19	20	-	12	8
MELD score		-	8.7±2.9	12.6±5.3*		-	8.4±2.4	11.2±3.5*
Serum albumin (g/dL)		-	3.8±0.5	3.2±0.6*		-	4.0±0.42	3.4±0.53*
Etiology (HCV/Alc/ HCV+Alc/NASH/ HBV/Others)†	45/24/ 15/16/ 3/6	-	27/7/ 7/12/ 2/4	18/17/ 8/4/ 1/2	20/10/ 2/30/ 24/7	-	11/3/ 0/17/ 15/4	9/7/ 2/13/ 9/3
PPI Use	55	10	19	26*	56	8	17	31
Lactulose†	38	0	0	38	19	0	0	19
Rifaximin†	31	0	0	31	8	0	0	8
Alcohol use†	17	10	5	2*	6	6	0	0*
NSBB†	44	-	15	29*	17	-	9	8
Prior Ascites	34	-	0	34*	24	-	0	24*
Prior HE†	40	-	0	40*	8	-	0	8*
Prior Variceal bleeding†	20	-	0	20*	8	-	0	8*
Prior jaundice†	2	-	0	2	14	-	0	14
Presence of Varices	41	-	9	32*	47	-	14	23*
Smoking	27	2	16	9*	24	13	9	12
Fiber supplement	7	0	4	3	3	0	0	5
Total daily caloric intake		2361±801	2461±1201	2260±740		2373±995	2399±890	2292±1015

% calorie protein		16.5±4.6	15.9±5.3	15.7±6.2		16.1±5.2	17.2±9.4	14.9±8.3
% calories fats		30.3±7.6	33.1±5.9	32.1±8.2		29.6±8.9	30.1±6.7	31.2±7.4
% calories carbohydrates		48.1±9.8	49.4±6.0	46.8±12.1		49.2±11.6	47.9±9.6	45.7±6.1
Shannon diversity index†		7.31±1.05	6.86±1.23	6.02±1.52*		7.83±0.91	8.04±0.30	7.86±0.97

Comp: compensated, decomp: decompensated, MELD: model for end-stage liver disease, HCV: hepatitis C virus, Alc: alcoholic liver disease, NASH: non-alcoholic steatohepatitis, PPI: proton pump inhibitor, NSBB: non-selective beta-blockers, HE: hepatic encephalopathy.

Data are presented as subject numbers or as mean±standard deviation.

†:p<0.05 between USA and Turkey, *p<0.05 within the same population.

Table 2: Specific Plasma Metabolites Driving the Differences Between and Within Cohorts from VIP scores in PLS-DA (Q2>0.40).

Metabolite	Turkey vs USA		Within Turkey		Within USA
	Control	Decomp	Control vs comp	Ctrl vs decomp	Ctrl vs decomp
Lactate (1.34-1.32ppm)	↑	↑	↑	↑	-
Lipid CH ₂ (1.32-1.22ppm)	↓	↑	↑	↑	↑
Lipid CH ₃ LDL (0.86-0.78ppm)	↑	↑	-	↑	↑
Lipid CH ₃ VLDL (0.92-0.86ppm)	↓	-	↑	↑	↑
Glucose (5.25-5.22ppm)	↓	↓	↓	↓	↓
Phosphocholine (3.22ppm)	↑	-	↑	↑	↑

↓: Lower in the group/cohort mentioned first compared to the one mentioned second,

↑: Higher in the group/cohort mentioned first compared to the one mentioned second,

-: similar between groups.

Figure Legends

Figure 1. Linear discriminant analysis (LEFSe) of predicted gut microbial function derived through PiCRUST (Phylogenetic Investigation of Communities by Reconstruction of Unobserved States) within corresponding subjects between countries. X axes represent the relative linear discriminant value of a predicted function that separates the groups that are represented by red or green.

A. USA controls (green bars) and Turkey controls (red bars); B. Compensated Cirrhosis: USA (green) vs Turkey (Red); C. Decompensated Cirrhosis: USA (green) vs Turkey (Red).

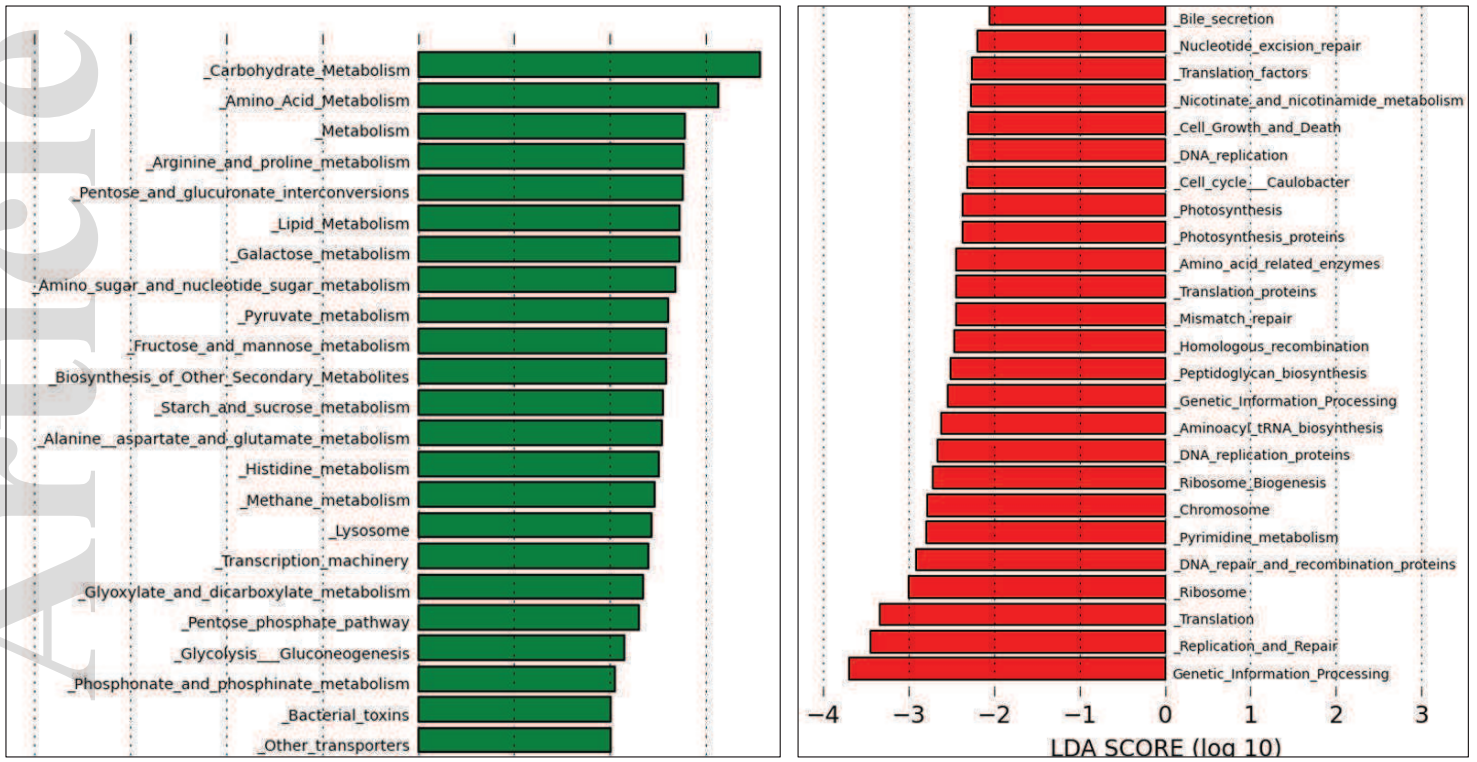
Figure 2. Linear discriminant analysis (LEFSe) of predicted gut microbial function derived through PiCRUST (Phylogenetic Investigation of Communities by Reconstruction of Unobserved States) within corresponding subjects between countries. X axes represent the relative linear discriminant value of a predicted function that separates the groups that are represented by red or green.

2A. USA cohort: compensated (red) vs decompensated cirrhosis (green); 2B. Turkish cohort: compensated (red) vs decompensated cirrhosis (green).

Figure 3. Plasma NMR metabolomics analysis showing orthogonal partial least squares discriminant analysis (OPLS-DA) results. 3A: USA vs Turkey according to clinical groupings. 3B: Compensated versus decompensated cirrhosis groupings according to country.

Figure 4. Correlation network differences between US and Turkey in cirrhosis patients using plasma metabolomics and gut microbial genera. Changes between correlation networks at $p < 0.001$ are shown. Nodes are coded as: purple (bacterial genera), green triangles (lipids), blue diamonds (amino acids). Edges are coded as: brown (gain in negative from Turkey to USA), red (positive in turkey and negative in USA), light blue (negative in Turkey and positive in USA), dark blue (gain in positive from Turkey to USA). 4A-D. Sub-networks from Correlation Network Differences Between American and Turkish Cohorts of Cirrhosis.

1B. Compensated Cirrhosis: USA (green) vs Turkey (Red)



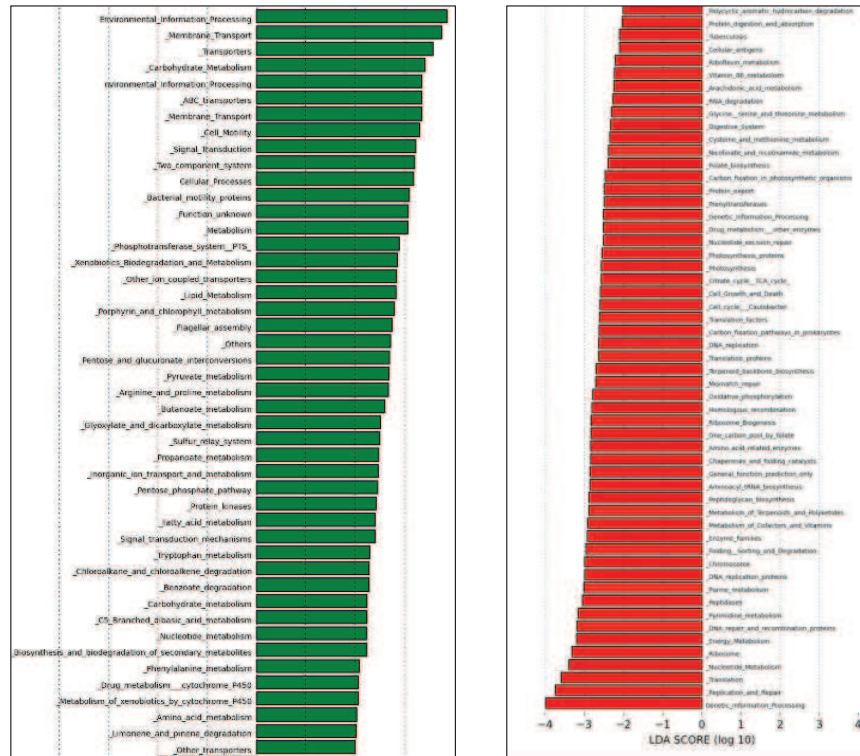
Linear discriminant analysis (LEFSe) of predicted gut microbial function derived through PICRUST (Phylogenetic Investigation of Communities by Reconstruction of Unobserved States) between countries. Comparison between USA (green bars) and Turkey compensated cirrhosis (red bars)

liv_14256_f1b.eps

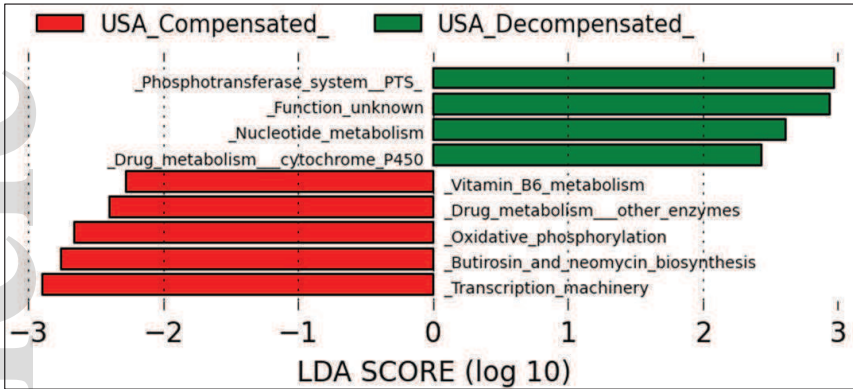
1C. Decompensated Cirrhosis: USA (green) vs Turkey (Red)

Linear discriminant analysis (LEFSe) of predicted gut microbial function derived through PiCRUST (Phylogenetic Investigation of Communities by Reconstruction of Unobserved States) in within corresponding subjects between countries. X axes represent the relative linear discriminant value of a predicted function that separates the groups that are represented by red or green.

Comparison between USA decompensated cirrhosis (green bars) and Turkey decompensated cirrhosis (red bars)

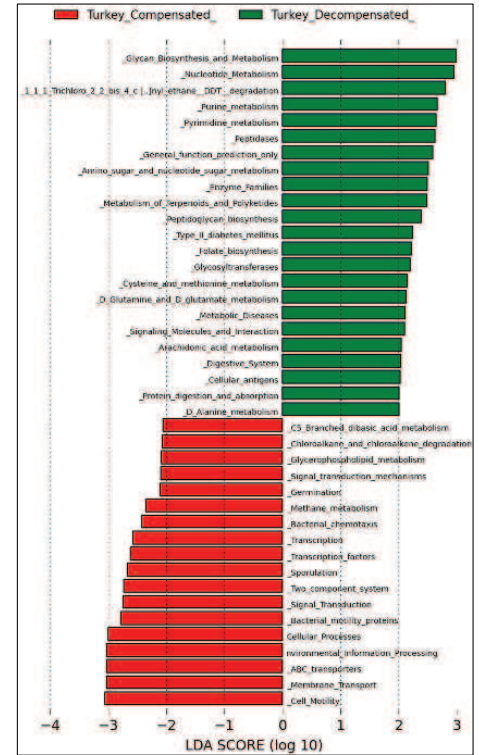


liv_14256_f1c.eps



2A: USA cohort: compensated (red) vs decompensated cirrhosis (green)

Linear discriminant analysis (LEFSe) of predicted gut microbial function derived through PiCRUST (Phylogenetic Investigation of Communities by Reconstruction of Unobserved States) in within corresponding subjects between countries. X axes represent the relative linear discriminant value of a predicted function that separates the groups that are represented by red or green.

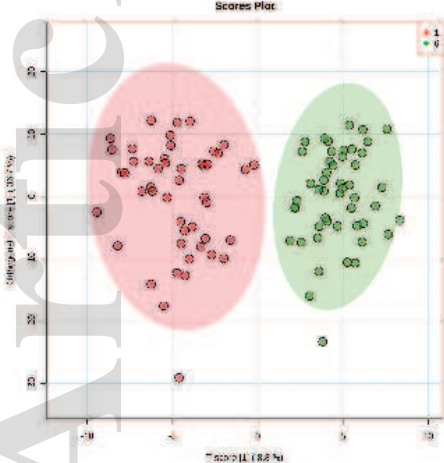


2B: Turkish cohort: compensated (red) vs decompensated cirrhosis (green)

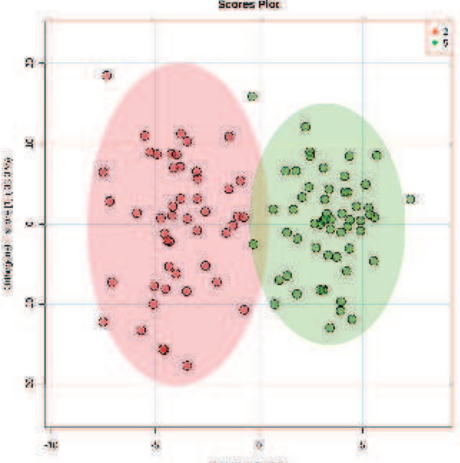
liv_14256_f2.eps

3A: OPLS-DA comparing USA vs Turkey according to clinical groupings

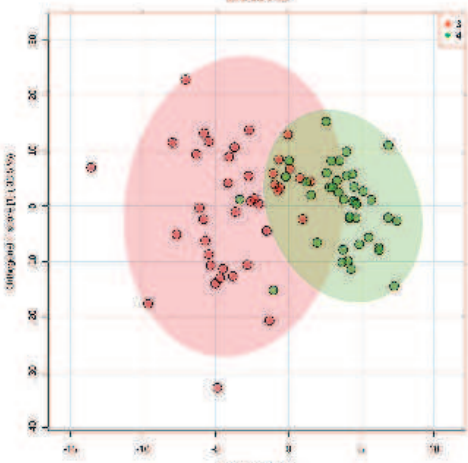
Controls
USA (red) – Turkey (green)



Compensated cirrhosis
USA (red) – Turkey (green)

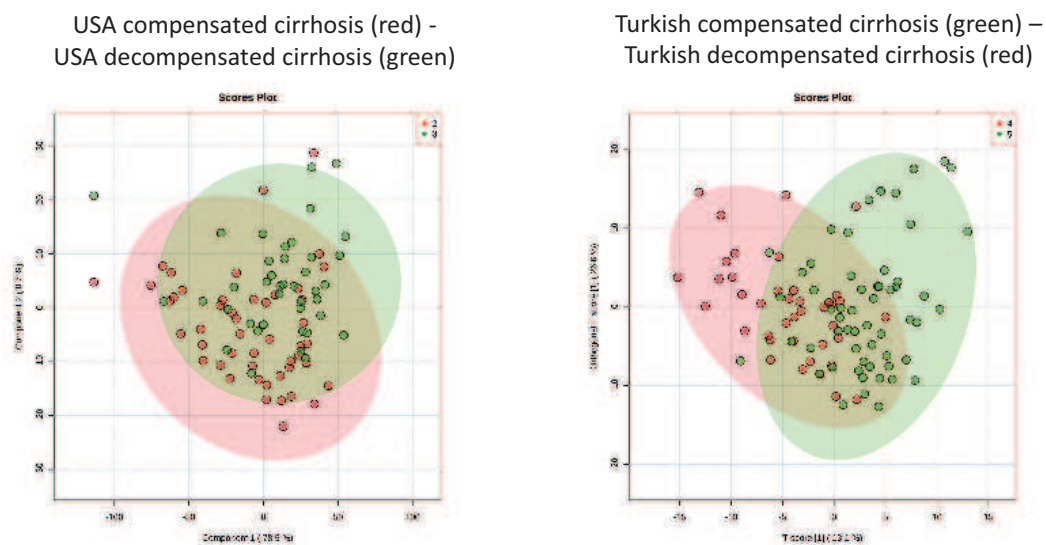


Decompensated cirrhosis
USA (red) – Turkey (green)

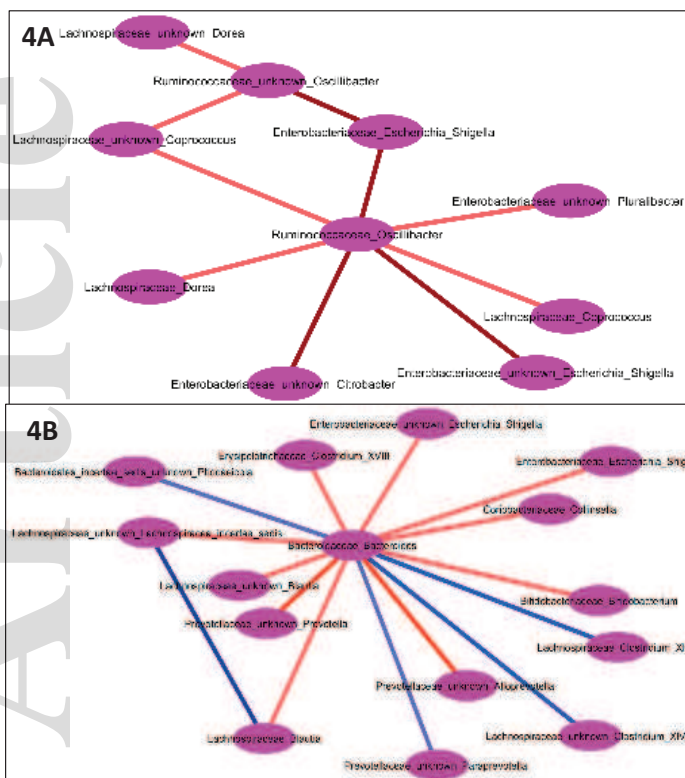


liv_14256_f3a.eps

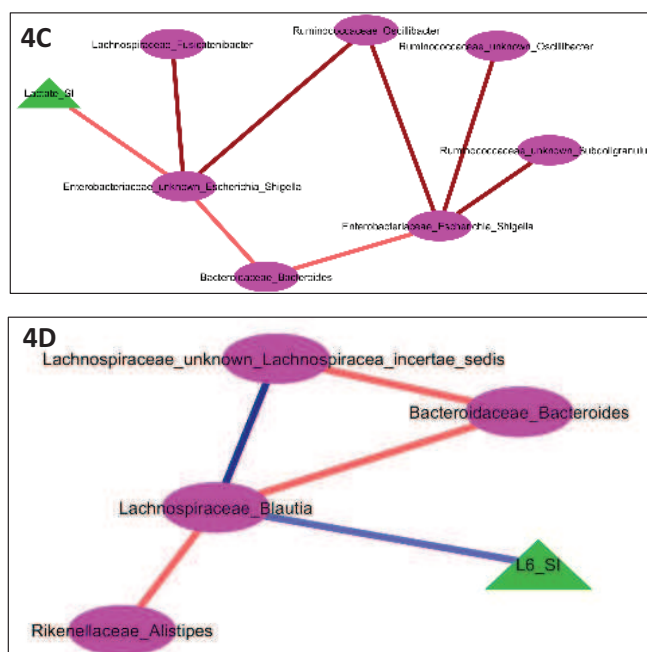
3B: OPLS-DA comparing cirrhosis groupings according to country



liv_14256_f3b.eps



Sub-networks from Correlation Network Differences Between American and Turkish Cohorts of Cirrhosis



Correlation network differences between US and Turkey in cirrhosis patients using plasma metabolomics and gut microbial genera. Changes between correlation networks at $p < 0.001$ are shown. Nodes are coded as: Purple are bacterial genera, Green triangles are lipids and blue diamonds are amino acids. Edges are coded as: Brown= gain in negative from Turkey to USA, Red= Positive in turkey and negative in USA, Light Blue= Negative in Turkey and positive in USA, Dark Blue= gain in positive from Turkey to USA.

liv_14256_f4.eps