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The gut microbiota and their metabolites in human arterial stiffness

Short title: The gut microbiome and ambulatory arterial stiffness index

Abstract

Gut microbiota-derived metabolites, such as short-chain fatty acids (SCFAs) have vasodilator properties in animal and human *ex vivo* arteries. However, the role of the gut microbiota and SCFAs in arterial stiffness in humans is still unclear. Here we aimed to determine associations between the gut microbiome, SCFA and their G-protein coupled sensing receptors (GPCRs) in relation to human arterial stiffness. Ambulatory arterial stiffness index (AASI) was determined from ambulatory blood pressure monitoring in 69 participants from regional and metropolitan regions in Australia (55.1% women, mean±SD 59.8±7.26 years of age). The gut microbiome was determined by 16S rRNA sequencing, SCFA levels by gas chromatography, and GPCR expression in circulating immune cells by real-time PCR. There was no association between metrics of bacterial α and β diversity and AASI or AASI quartiles in men and women. We identified 2 main bacteria taxa that were associated with AASI quartiles: *Lactobacillus* spp. was only present in the lowest quartile, while *Clostridium* spp. was present in all quartiles but the lowest. AASI was positively associated with higher levels of plasma, but not faecal, butyrate. Finally, we identified that the expression of GPR43 (*FFAR2*) and GPR41 (*FFAR3*) in circulating immune cells were negatively associated with AASI. In conclusion, this suggests that arterial stiffness is associated with lower levels of the metabolite-sensing receptors GPR41/GPR43 in humans, blunting its response to BP-lowering metabolites such as butyrate. The role of *Lactobacillus* spp. and *Clostridium* spp., as well as butyrate-sensing receptors GPR41/GPR43 in human arterial stiffness needs to be determined.

Keywords: arterial stiffness, pulse wave velocity, metagenome, short-chain fatty acids, metabolites

Introduction

Vascular dysfunction, most notably arterial stiffness, represents a vital preclinical stage in the development of cardiovascular disease. Arterial stiffness is typically defined as reduced distensibility of the arterial wall (1), resulting in gradual loss of elastic compliance and thus, stiffening (2). The gut microbiota is the community of microorganisms that inhabit the intestine (3,4). Perturbations to the composition of the gut microbiota are known as gut dysbiosis (5). Recent data have indicated that gut dysbiosis elicit inflammatory responses and oxidative stress on metabolically active tissues such as the vasculature (6,7).

The human gut microbiota has been associated with blood pressure (BP) in several human studies.(3,4,8) Experiments using germ-free animal models have shown that the gut microbiota is not merely associated with BP, but it indeed can increase BP (9,10). A possible mechanism involves gut microbial-derived metabolites, such as short-chain fatty acids (SCFAs), which are produced during the fermentation of certain types of dietary fibre by intestinal bacteria (3). We and others have demonstrated that treatment with the three main SCFAs, acetate, propionate and butyrate, lowers BP and reduces total peripheral resistance in mice (9,11). Relevant to arterial stiffness, acetate, propionate and butyrate have vasodilator effects in human (12) and mouse *ex vivo* arteries (13,14). While there is increasing evidence for a role of the gut microbiota in arterial stiffness, this remains poorly understood in humans. This knowledge could represent new therapeutic opportunities to reduce arterial stiffness, such as being studied in human hypertension (15).

Ambulatory arterial stiffness index (AASI) is a measure of arterial stiffness calculated from ambulatory BP monitoring (16). Here we aimed to study the relationship between arterial stiffness in humans, measured as AASI, and the gut microbiome, their metabolites and receptors.

Methods

Participants and recruitment

The cohort was recently described elsewhere (17). A total of 69 participants (40 in a metropolitan clinic and 29 in a regional clinic) untreated for hypertension were recruited between October-2016 and April-2018. Probiotic or antibiotic use in the past 3 months were used as exclusion criteria, among others. This study complied with the Declaration of Helsinki, and was approved by the human research ethics committee of the Alfred Hospital (approval 415/16, registration ACTRN12620000958987). All participants provided informed consent.

Blood pressure measurement and hypertension diagnosis

Participants were fitted with a calibrated ambulatory BP monitoring device (AND or SpaceLabs) for 24-hours. AASI values were automatically derived from the regression slope of diastolic blood pressure (DBP) and systolic blood pressure (SBP) using unfiltered 24-h recordings, following the formula: AASI=1–slope (DBP / SBP). The cohort was separated into quartiles (Q1: <0.365; Q2: 0.365–0.504; Q3: 0.504–0.581; Q4: >0.581), top 50% (>0.504) and bottom 50% values (<0.504) of the AASI distribution.

Faecal DNA extraction, library preparation and sequencing

This study followed guidelines for gut microbiota studies in hypertension (18) and the Strengthening The Organization and Reporting of Microbiome Studies (STORMS) reporting (19) (available at (17)). Sample collection, DNA extraction and library preparation and sequencing were described in detail previously (17). Briefly, the V4-V5 region of the bacterial 16S rRNA was amplified by PCR, and were sequenced in an Illumina MiSeq sequencer (300bp paired-end reads). To increase the reproducibility of the findings, all

samples were independently sequenced twice. These technical duplicated samples were combined for the analyses described below. Microbiome data is publicly available at the NCBI Sequence Read Archive database at https://dataview.ncbi.nlm.nih.gov/object/PRJNA722359?reviewer=qu9taron24c26mcfvgk30qf56g.

Bioinformatic analyses of gut microbiome

Sequence reads from samples were first analysed using the QIIME2 framework (20) as we reported recently (17), trained against the SILVA database (version 138) 99% OTU reference sequences specific for bacterial V4-V5 rRNA regions. β diversity metrics were generated from the rarefied samples, including unweighted and weighted Unifrac metrics shown as Principle Coordinate Analysis (PCoA) plots. Linear discriminant analysis (LDA) effect size (LEfSe) (21) was used to identify differentially abundant taxa between groups, with a specified effect size cut-off of 2.0 and Kruskal-Wallis test $P<0.05$. This data was validated using edgeR differential abundance analysis (false discovery rate adjusted $P<0.05$ on species) on MicrobiomeAnalyst (22,23). Further analyses were performed on MicrobiomeAnalyst from the rarefied samples, including α diversity, abundance profiling and clustering analysis. Features with a minimum of four counts occurring at a prevalence of 10% in samples were included. One participant was excluded from all analyses due to low total number of sequencing reads (<10,000). Data was scaled using the Total Sum Scaling (TSS) normalisation method to account for technical bias associated with varying sequencing depths in different libraries (24).

Short-chain fatty acids measurement

Briefly, plasma SCFAs were measured in 200 µL and faecal SCFAs were measured from 1 g of faecal sample, all in triplicates, as previously published, in an Agilent GC6890 coupled to

a flame-ionisation detector (25,26). A coefficient of variation of <10% within triplicate samples was used as a quality control measure.

Blood expression of SCFA receptors and transporters

As previously explained (17), we quantified the expression of the mRNA of the three main SCFA-sensing receptors GPR41 (*FFAR3),* GPR43 (*FFAR2*) and GPR109A (*HCAR2*) in circulating immune cells (which highly express them (27)) in 50 participants by real-time PCR (qPCR). TaqMan assays were used (17), with glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*) and β-actin (*ACTB*) as housekeeping genes. All expression experiments were run in duplicates and significance was assessed by $2^{-\Delta\Delta C_T}$ method.

Statistical analyses

Data were analysed blind. GraphPad Prism (version 8) package was used for graphing, and SPSS for Windows (release 25) for statistical analyses. Non-parametric tests were used in the case of non-normally distributed data. One-way ANOVA was performed on AASI quartiles, while a two-tail independent sample t-test was used to compare AASI data between two groups (top/bottom 50%; Q1 versus Q2–Q4 of the AASI distribution). α diversity score correlations with AASI values were performed using Spearman's correlation coefficients. Further analyses were conducted using step-wise multiple linear regression models for acetate, butyrate, propionate, *GPR41*, *GPR43* and *GPR109A* levels. These models had clinical (age, sex, body mass index, overall mean arterial pressure [MAP]) variables as independent parameters (criteria of F-entry probability: 0.15, removal: 0.20). Data are presented as mean±SD unless otherwise specified, and those with a $P<0.05$ considered significant.

Results

Baseline characteristics

Table 1 summarises the baseline characteristics of the 69 participants included in this study across quartiles, top 50% and bottom 50% of the AASI distribution. As expected, participants in the top 50% of the AASI distribution had significantly higher AASI scores ($P< 0.001$). No significance was found between quartiles, as well as top and bottom 50% of the AASI distribution for all other independent variables listed in Table 1.

Gut microbiome

A total of over 4.3 million reads were denoised, merged and underwent chimera filtering, resulting in an average read count of 63,000 per sample. Samples were rarefied to 29,000 reads to allow for consistent and plateauing diversity metrics (Figure S1), achieved with the exclusion of data from one participant (thus, total 68 participants were included). Two frequently used metrics for predicted gut microbiome studies were measured; α- and β-diversity. We found no association between four metrics of α-diversity (observed OTUs, Chao1 index, Shannon index, Simpson index) and AASI (Figures 1, 2 and S3). These findings were validated in correlations between α-diversity and AASI as a continuous measurement (Figure 2), as well as in regression analyses adjusted by sex, age, BMI and MAP (data not shown, all $P>0.9$). Due to numerous studies having explored sexual dimorphism in arterial stiffening, as well as one report finding an association between α-diversity and arterial stiffness (28), we also performed a separate analysis between α-diversity and AASI scores in female participants only (Figure S4). Consistent with our findings in the complete cohort, we did not observe any significant correlations. Similar results were observed when assessing β-diversity of the entire cohort; both weighted and unweighted UniFrac distances showed no significant clustering patterns of either AASI as a continuous

variable (Figure 3A, B) or quartiles (Figure 3C, D). Furthermore, as expected, phylum-level taxa plots of the quartiles and top 50% and bottom 50% of AASI distribution showed that the overall relative abundance of taxa belong to the two main phyla Firmicutes and Bacteroidetes (Figure 4). We explored this further by performing LEfSe in order to identify potential taxa that were more prevalent in one group compared to another, shown by LDA scores higher than 2.0 (Figure 5). This cut-off was specifically chosen to allow for the identification of taxa that were likely to have biological significance as opposed to just a significant *P*-value. We found that two specific taxa, *Lactobacillus spp.* and *Clostridium spp.*, were differentially abundant between the top 50% and bottom 50% of the AASI distribution, as well as between Q1 versus Q2, Q3 and Q4. These findings were validated through edgeR differential abundance analysis, which allowed for the exploration of the quartiles of the AASI distribution, which we could not do using LEfSe (Figure 6). We identified that participants within Q1 of the AASI distribution had a greater abundance of *Lactobacillus*, *Lachnoclostridium* and *Ruminoclostridium* compared to participants with AASI values within the three remaining quartiles. Interestingly, several uncultured species of *Clostridium* were found to be significantly more abundant in participants with AASI values within Q2-Q4 compared to participants with AASI values in Q1 (Figure 6).

Short-chain fatty acids and receptors

We then studied the levels of SCFAs in the circulation and in faecal samples in relation to AASI. There was a positive, albeit not significant, correlation between plasma butyrate (r=0.20, *P*=0.131) and AASI. We then performed a sensitivity analysis, which showed that both plasma acetate (β=-0.001±0.001, *P*=0.024) and butyrate (β=0.033±0.012, *P*=0.008) remained significant, as well as BMI and sex. However, butyrate had the largest impact on AASI, evidenced as standardised β (0.42), compared to all other variables (-0.31 to -0.37). All faecal SCFAs were not associated with AASI (data not shown). We then analysed the

expression of the SCFA receptors GPR41 (*FFAR3*), GPR43 (*FFAR2*) and GPR109A (*HCAR2*) in circulating immune cells in relation to AASI. We identified that *FFAR2* (r=-0.373, P=0.011) and *FFAR3* (r=-0.300, P=0.043) were negatively correlated with AASI, but and *HCAR2* (r=-0.222, P=0.148) were not significant.

Discussion

Through the combination of gut microbiome sequencing, metabolite and receptor quantification, we were able to uncover novel relationships with arterial stiffness, shown as AASI. In particular, we identified that AASI was associated with lower abundance of *Lactobacillus spp.* and higher abundance of several species from the genus *Clostridium.* Moreover, to our knowledge, this is the first study to assess SCFAs and GPCRs in relation to human arterial function. Specifically, our results demonstrated a small but positive correlation between plasma butyrate levels and AASI scores independent of conventional risk factors (e.g. sex, BMI, MAP), but reduced levels of the main receptors that sense these metabolites, GPR41 and GPR43, in circulating immune cells.

A growing body of evidence supports the gut microbiota has a causal role in experimental and human hypertension (3,4). Thus, a relationship between the gut microbiota and arterial stiffness, a preclinical marker of cardiovascular disease, would be expected. Gut microbiota contributes to the onset of pro-inflammatory pathways, affecting metabolic tissues including, but not limited to, the vasculature (6,29), suggesting a microbial compositional difference between lower and higher degrees of arterial stiffness. Moreover, sex-specific differences in arterial stiffness have also been reported (2,30). Particularly relevant for our findings is the report of an inverse association between gut microbiome α-diversity and arterial stiffness, measured by pulse wave velocity, in women (31). However, we were not

able to replicate this association between microbial α-diversity and AASI scores in our cohort. We cross-validated these findings with female-only participants to potentially identify a sex-specific difference in microbial diversity, and obtained similar results. This may be due to the difference in the technique used to assess arterial stiffness between studies (31). Our findings may suggest that microbial pathways, instead of major microbiota dysbiosis, are more relevant to arterial stiffness, as we reported in hypertension (17).

Despite this, we were able to identify some differentially prevalent bacteria taxa between participants with lower versus higher AASI. Especially important are the greater levels of *Lactobacillus* spp., a low abundance species commonly used in probiotics, observed in the lowest quartile of the AASI distribution. This is consistent with findings that high sodium intake in mice and humans reduced intestinal survival of *Lactobacillus spp.* and drove an increase in BP via *Lactobacillus*-derived metabolites (32). Contrastingly, we detected greater levels of several *Clostridium* spp. in the three highest AASI quartiles, while they were absent in the lowest AASI quartile. This is unsurprising, as an array of *Clostridium* spp. are positively associated in cardiovascular disease (33) and have been found to be amongst the more abundant bacterial species in hypertensive patients (34). This may explain the elevated levels of plasma butyrate found in participants with higher AASI values, as the main butyrate-producing bacteria are anaerobes, including those of the Clostridia family (35).

Plasma butyrate, the third most abundant SCFAs reported to have BP-lowering properties in mice (27), was found to be elevated in patients with higher AASI. However, it should be noted that this association was relatively small. Butyrate is sensed by three GPCRs, including GPR41 and GPR43 (9,27). We previously showed mouse models lacking GPR41 and GPR43 have increased cardiac fibrosis (9). Moreover, six-months-old GPR41 knockout mice have increased pulse wave velocity, and collagen and elastin deposition in their arteries (13). Importantly, GPR41/GPR43 are highly expressed in immune cells (27). When activated

through the binding of SCFAs, GPR41 and GPR43 promote anti-inflammatory downstream pathways (35,36), such as through the polarisation of T lymphocytes into T regulatory lymphocytes (37). These cells then migrate and accumulate in the renal cortex where they may elicit their BP-lowering properties (38). In our study, we found participants with higher AASI values had lower levels of the SCFA-sensing receptors GPR41 and GPR43 in circulating immune cells, which may blunt their response to BP-lowering metabolites. This may also explain why we observed an increase in plasma butyrate, as it is unable to bind to its receptors to elicit its reported vasodilatory effects on the vasculature (12). Moreover, deficiency in GPR41/GPR43 signalling can lead to immune dysfunction and a shift from an anti- to pro-inflammatory phenotype, evidenced through enhanced peripheral neutrophil-to-lymphocyte ratios in hypertensive patients (39). Thus, the deficiency of GPR41/GPR43 signalling may be partially responsible for the pro-inflammatory phenotype, which in turn affects vasculature and increases arterial stiffness.

We acknowledge that there are some limitations to our study, including the relatively small sample size. However, our samples are well-characterised with ambulatory BP monitoring as well as SCFA and GPCR quantification. Due to the small sample size, our results need to be independently validated, preferably through meta-analysis. However, this will be difficult to achieve due to lack of gut microbiota studies with ambulatory BP monitoring data available. Despite this, our study took advantage of the only multi-site cohort published to date that has AASI reported in both men and women who were all untreated for hypertension. We also acknowledge that carotid-to-femoral pulse wave velocity would be a more suitable measurement of arterial stiffness. However, AASI has been validated as a surrogate marker of pulse wave velocity and arterial stiffness (16).

Conclusion

Arterial stiffness, measured as AASI, was not associated with gut microbiota α-diversity. However, we identified associations between the abundance of specific taxa, namely lower *Lactobacillus* spp. and higher *Clostridium* spp., and AASI. Moreover, it was found that higher levels of the SCFA butyrate was up-regulated in participants with higher AASI values. The immune expression of butyrate's main sensing receptors, GPR41/GPR43, were negatively associated with AASI. This suggests that specific microbial taxa may impact human vascular function via production of SCFAs and sensing via their receptors, as well as their downstream inflammatory pathways.

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Disclosures

None.

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Figure Legends

Figure 1. α-diversity profiles and box plots of quartiles of the AASI distribution. α-diversity profiling and consequent box plots illustrating α-diversity indices of quartiles of the AASI distribution using A) observed OTUs (*P*=0.27), B) Chao1 (*P*=0.26), C) Shannon metrics (*P*=0.91), and D) Simpson indeces (*P*=0.91). Box plot data presented as median and inter-quartile range (IQR).

Figure 2. α-diversity score correlation analyses with AASI. α-diversity scores and AASI values showing Spearman correlation using A) observed OTUs, B) Chao1 index, C) Simpson index and D) Shannon index.

Figure 3. β-diversity principal coordinate analysis plots. β-diversity principal coordinate analysis plots of A) unweighted (i.e., microbial diversity based on presence/absence) and B) weighted (i.e., microbial diversity based on abundance) UniFrac analyses of AASI values and C) unweighted and D) weighted UniFrac analyses of quartiles of the AASI distribution.

Figure 4. Phylum-level taxa plots. Percentage of total bacteria presented at the phylum level in A) quartiles and B) top 50% and bottom 50% of the AASI distribution.

Figure 5. Predicted gut microbiome taxa linear discriminant analysis effect sizes. Predicted gut microbiome taxa that are different between A) top 50% and bottom 50% of the AASI distribution, B) Q1 versus Q2–Q4 at the genus level, and C) at the species level of Q1 versus Q2 – Q4 with a linear discriminant analysis (LDA) score of at least 2.

Figure 6. Differential abundance analysis of specific bacterial taxa using the edgeR algorithm. Differential abundance analysis of *Lactobacillus spp.* and *Clostridium spp*. between quartiles (A–D) and Q1 versus Q2–Q4 (E–J) of the AASI distribution. By default, relative log expression normalisation was performed on the data, and data presented is

presented as log-transformed count. False discovery rate adjusted *P*-value cut-off=0.05. Box plot data presented as median and IQR.

Tables

Table 1. Demographics and clinical characteristics of participants.

Variable	**Quartile 1 (< 0.365)**	**Quartile 2 (0.365–0.504)**	**Quartile 3 (0.504–0.581)**	**Quartile 4 (> 0.581)**	***P*-value**		**Top 50% of AASI (> 0.504)**	**Bottom 50% of AASI (< 0.504)**	***P*-value**
Sample size (n, %)	17, 24.6%	17, 24.6%	18, 26.1%	17, 24.6%			35, 50.7%	34, 49.3%	
Age (years)	59.0±7.14	58.6±8.72	58.3±6.85	62.5±6.05	0.319		60.4±6.71	58.9±7.93	0.401
BMI (kg/m^2)	25.4±2.74	25.6±2.61	25.6±2.91	24.1±3.02	0.486		24.8±3.01	25.5±2.63	0.324
Waist to hip ratio	0.85±0.077	0.89±0.069	0.86±0.088	0.87±0.11	0.472		0.87±0.075	0.86±0.096	0.673
24-hour MAP	87.6±6.43	90.1±10.3	89.8±12.3	94.1±10.5	0.389		91.9±11.5	88.9±8.54	0.221
Sex (% female)	70.6%	64.7%	44.4%	35.3%	0.891		54.3%	55.9%	0.894
AASI score	0.268±0.0911	0.443±0.0474	0.537±0.0232	0.705±0.0753	0.430		0.618±0.101	0.355±0.114	<0.001

Data are shown as mean±standard deviation or numbers and percentages. Legend: ambulatory arterial stiffness index, AASI; body mass index, BMI; waist to hip ratio.

Figures

Figure 1

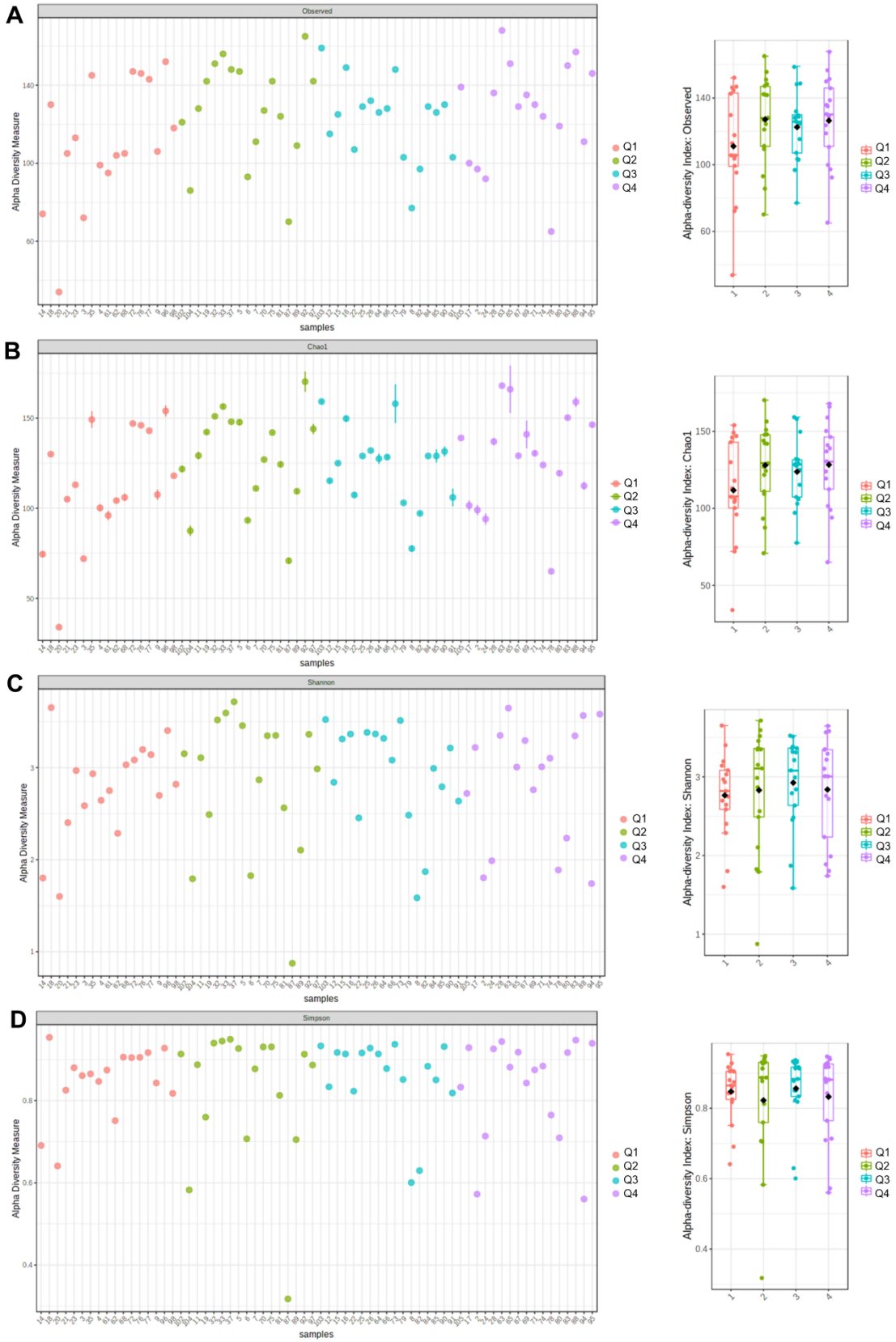


Figure 2

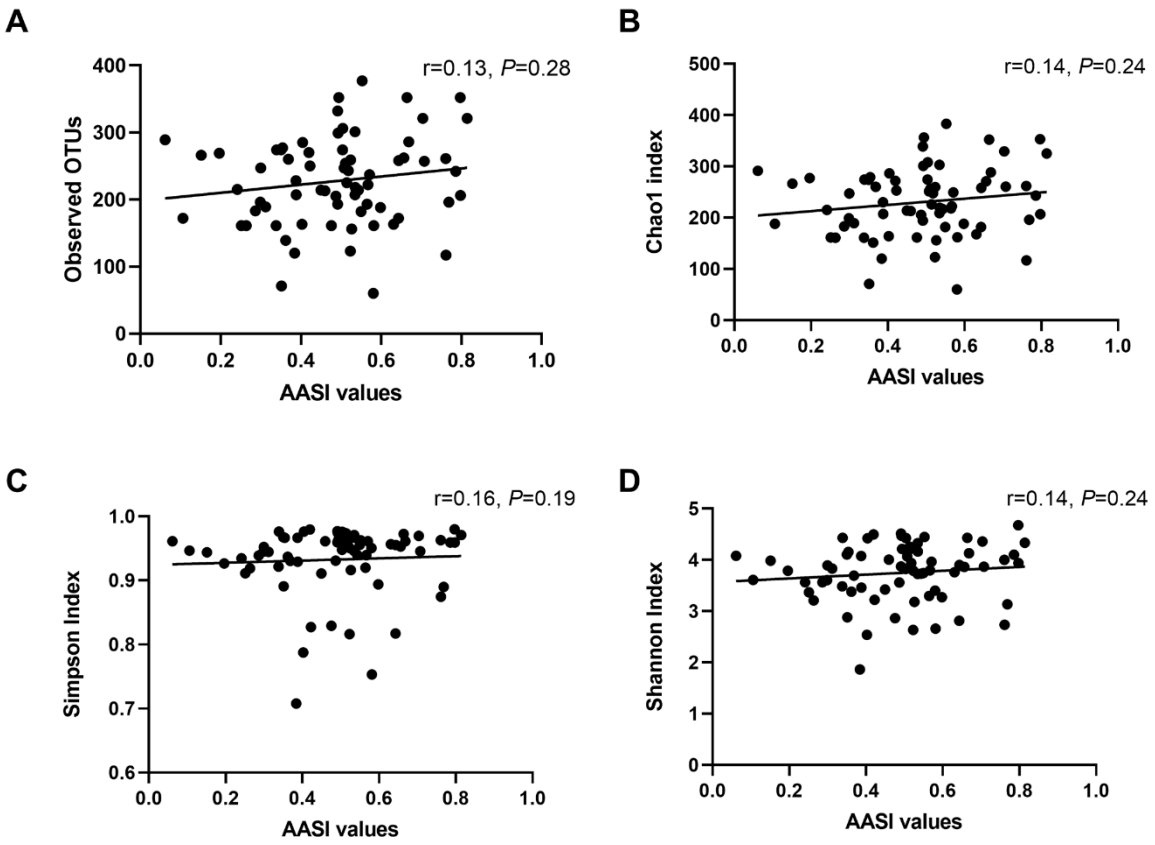

A
r=0.13, P=0.28
Observed OTUs
AASI values
B
r=0.14, P=0.24
Chao1 index
AASI values
C
r=0.16, P=0.19
Simpson Index
AASI values
D
r=0.14, P=0.24
Shannon Index
AASI values

Figure 3

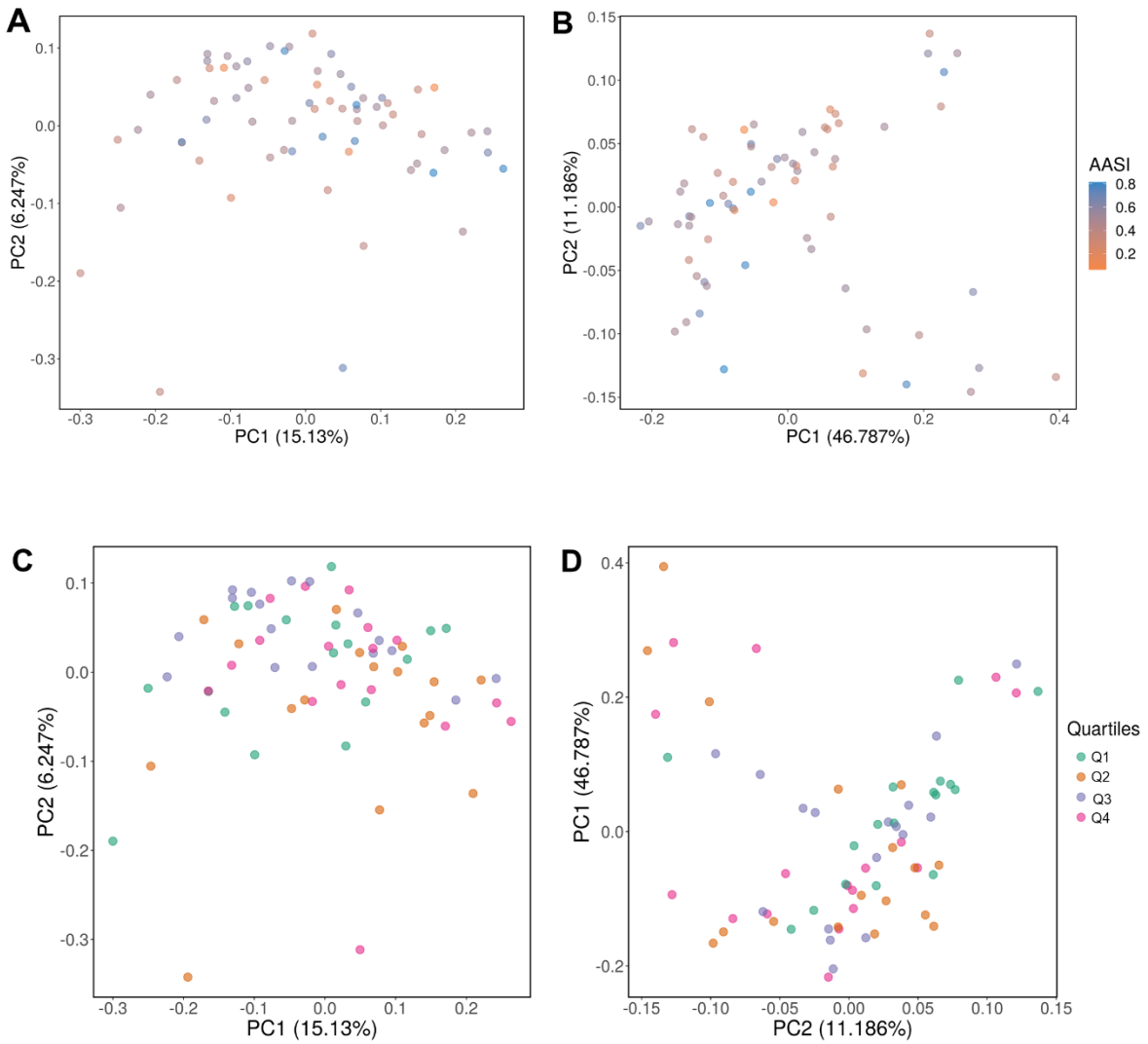

A
PC2 (6.247%)
PC1 (15.13%)
B
PC2 (11.186%)
PC1 (46.787%)
AASI
0.8
0.6
0.4
0.2
C
PC2 (6.247%)
PC1 (15.13%)
D
PC1 (46.787%)
PC2 (11.186%)
Quartiles
Q1
Q2
Q3
Q4

Figure 4

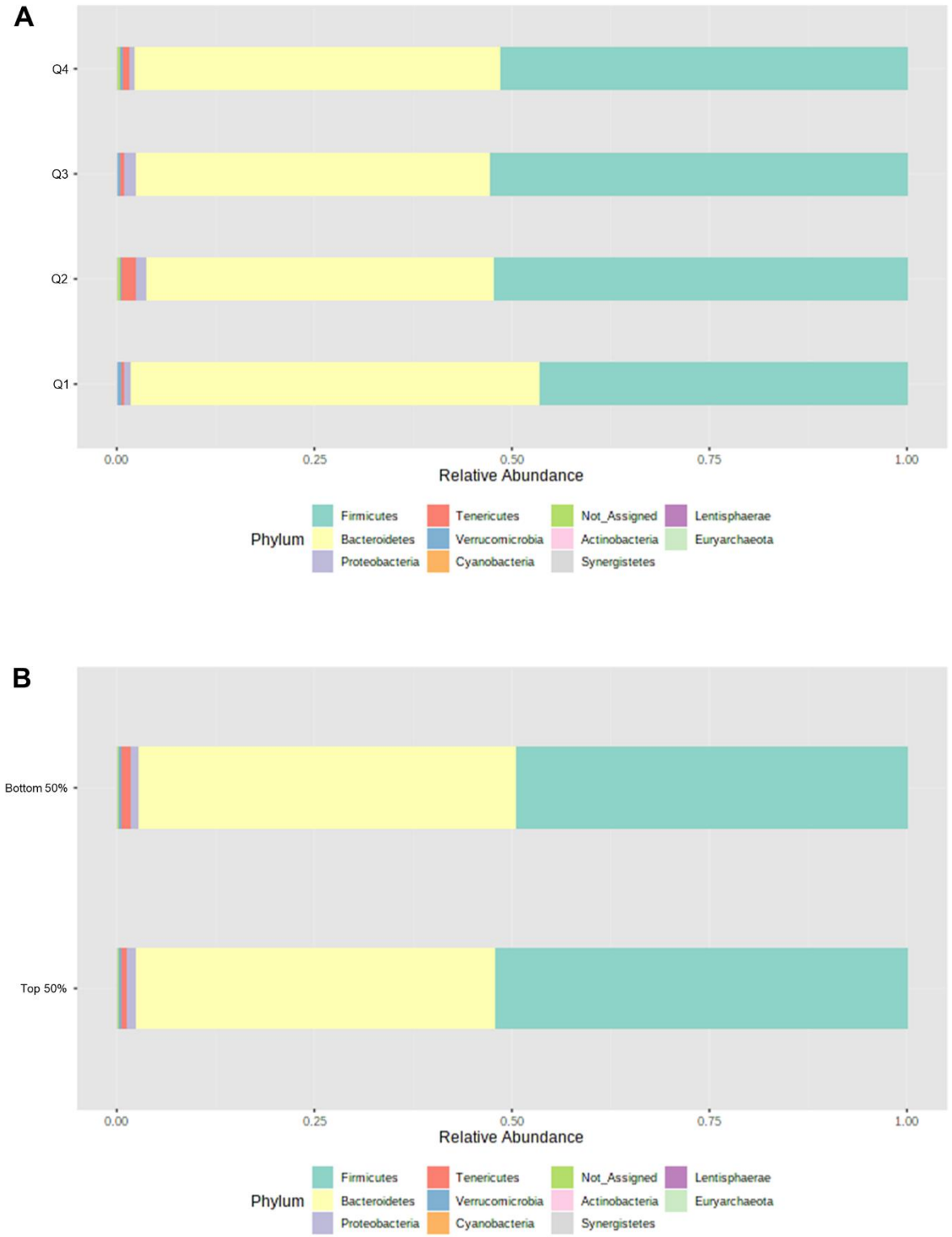

A
Q4
Q3
Q2
Q1
0.00
0.25
0.50
0.75
1.00
Relative Abundance
Phylum
Firmicutes
Bacteroidetes
Proteobacteria
Tenericutes
Verrucomicrobia
Cyanobacteria
Not_Assigned
Actinobacteria
Synergistetes
Lentisphaerae
Euryarchaeota
B
Bottom 50%
Top 50%
0.00
0.25
0.50
0.75
1.00
Relative Abundance
Phylum
Firmicutes
Bacteroidetes
Proteobacteria
Tenericutes
Verrucomicrobia
Cyanobacteria
Not_Assigned
Actinobacteria
Synergistetes
Lentisphaerae
Euryarchaeota

Figure 5

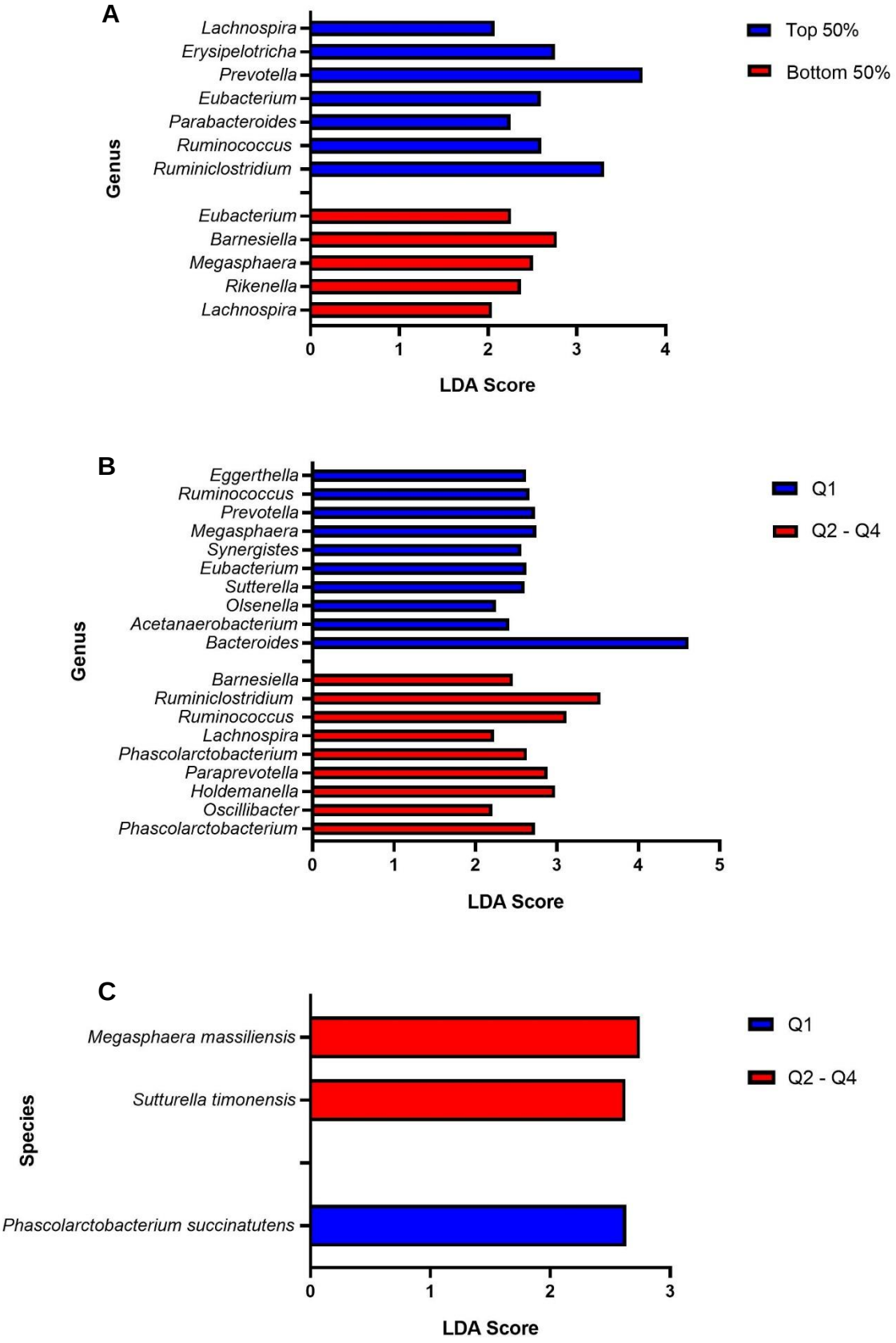
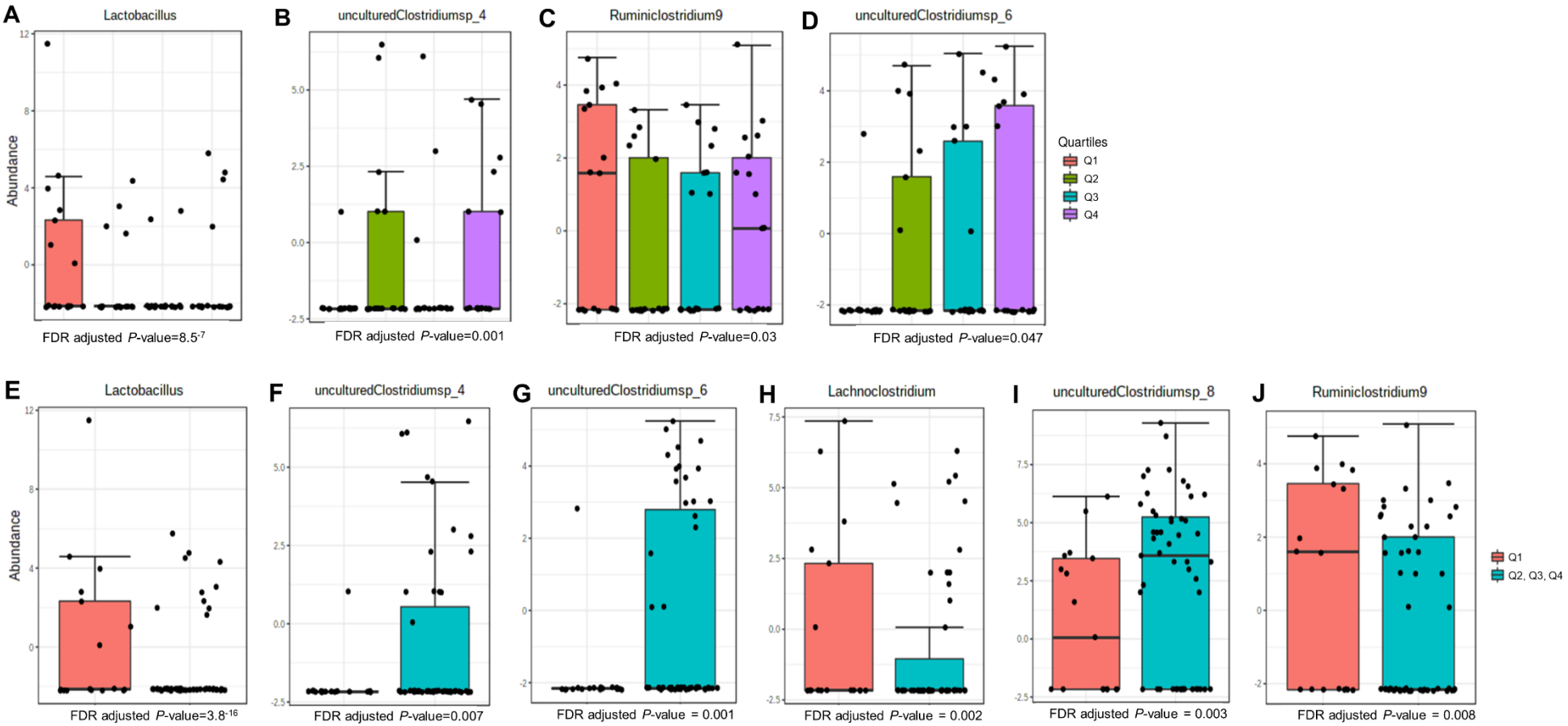

A
Lachnospira
Erysipelotricha
Prevotella
Eubacterium
Parabacteroides
Ruminococcus
Ruminiclostridium
Eubacterium
Barnesiella
Megasphaera
Rikenella
Lachnospira
Genus
0
1
2
3
4
LDA Score
Top 50%
Bottom 50%
B
Eggerthella
Ruminococcus
Prevotella
Megasphaera
Synergistes
Eubacterium
Sutterella
Olsenella
Acetanaerobacterium
Bacteroides
Barnesiella
Ruminiclostridium
Ruminococcus
Lachnospira
Phascolarctobacterium
Paraprevotella
Holdemanella
Oscillibacter
Phascolarctobacterium
Genus
0
1
2
3
4
5
LDA Score
Q1
Q2 - Q4
C
Megasphaera massiliensis
Sutturella timonensis
Phascolarctobacterium succinatutens
Species
0
1
2
3
LDA Score
Q1
Q2 - Q4

Figure 6


A
Lactobacillus
Abundance
FDR adjusted P-value=8.5-7
B
unculturedClostridiumsp_4
FDR adjusted P-value=0.001
C
Ruminiclostridium9
FDR adjusted P-value=0.03
D
unculturedClostridiumsp_6
FDR adjusted P-value=0.047
Quartiles
Q1
Q2
Q3
Q4
E
Lactobacillus
Abundance
FDR adjusted P-value=3.8-16
F
unculturedClostridiumsp_4
FDR adjusted P-value=0.007
G
unculturedClostridiumsp_6
FDR adjusted P-value = 0.001
H
Lachnoclostridium
FDR adjusted P-value = 0.002
I
unculturedClostridiumsp_8
FDR adjusted P-value = 0.003
J
Ruminiclostridium9
FDR adjusted P-value = 0.008
Q1
Q2, Q3, Q4