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## Investigating intestinal permeability and gut microbiota roles in acute coronary syndrome patients\*

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### Abstract

**Background:** Acute Coronary Syndrome (ACS) is a leading cause of morbidity and mortality. Perturbed gut- microbiota (dysbiosis) and increased intestinal permeability (leaky-gut) with translocation of bacterial antigens, play critical role in obesity and metabolic syndrome, which are also major ACS risk factors. Additionally, Trimethylamine-N-Oxide (TMAO), a metabolite produced by phylum Proteobacteria in gut is implicated in developing ACS. As Proteobacteria is a major source of translocated antigen lipopolysaccharides (LPS), we hypothesized that ACS patients have leaky-gut condition characterized by dysbiosis with increased Proteobacteria, leading to elevated blood levels of TMAO and LPS.

**Methods:** In a pilot case-control study, we enrolled 19 ACS patients (within 72-h of cardiac events) and 19 healthy-controls. Gut barrier function was determined using lactulose-to-mannitol urinary excretion ratio (L/M ratio). Stool microbiome composition was examined using 16S

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\***Note:** This study was conducted and completed at the University of New Mexico (UNM) and corresponding author left UNM to pursue clinician career at Promedica Digestive Health Care/University of Toledo, Toledo, Ohio

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<sup>8</sup>.Declarations

Ethics approval and consent to participate

The study was approved by the HRPO Human Research Protection Office and IRB (internal review board) at the University of New Mexico (UNM) Health Science Center, study protocol 13–605. All participants signed an informed consent to participate in study and a Health Insurance Portability and Accountability Act (HIPPA) compliant waiver form.

Competing interests

There is no conflict of interest by any of the authors of this study.

Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.humic.2019.100059>.

sequencing and predictive functional analysis for LPS biosynthesis pathway by PICRUSt tool. Serum TMAO and LPS levels were measured.

**Results:** ACS patients had increased Gammaproteobacteria compared to controls:  $1.8 \pm 3.0$  vs.  $0.2 \pm 0.4\%$  ( $P = 0.04$ ). Though Proteobacteria level was increased but not statistically significant:  $4.1 \pm 3.8$  vs.  $2.1 \pm 1.7\%$  ( $P = 0.056$ ). L/M-ratio was three times higher in ACS patients;  $0.06 \pm 0.07$  vs  $0.023 \pm 0.02$ , ( $P = 0.014$ ). Surprisingly, there was no difference in the mean serum LPS or TMAO levels. However, PICRUSt analysis indicated increased Proteobacteria population increasingly contributed to LPS biosynthesis in ACS patients only.

**Conclusions:** ACS patients likely to have leaky-gut and perturbed gut microbiota. Further studies are required to precisely define the role of dysbiosis in ACS.

## Keywords

Dysbiosis; Intestinal permeability; Proteobacteria; TMAO; Endotoxins; Acute coronary syndrome

## 1. Introduction

Acute Coronary Syndrome, (ACS; *aka* heart attack) is a leading cause of morbidity and mortality. Despite decades of research, our understanding of ACS pathophysiology is still obscure, especially in the context of the gut-microbiome. The gut-microbiome is increasingly recognized as a 'super organ' that serves plethora of important host functions such as metabolism and immune homeostasis [1]. These functions are attributed to bacterial genomic content of gut-microbiome which outnumbers our own genomic content by about 150 times [2]. Therefore, it is not surprising that alterations in the intestinal microbiome *aka* 'dysbiosis' are associated with various disease conditions, including metabolic syndrome, atherosclerosis and obesity; conditions which are also major ACS risk factors [3].

The gut dysbiosis is associated with increased intestinal permeability '*aka* leaky gut' [4]. Leaky gut is implicated in cardiovascular complications [5] besides, pathophysiology of Celiac disease, Inflammatory Bowel Disease (IBD) [6]. Furthermore, translocation of luminal antigens to the systemic circulation (with underlying leaky gut) has also been postulated to be an important pathogenic factor in non-alcoholic fatty liver disease, obesity, Type 2 diabetes, and metabolic syndrome, all of which are also known risk factors for ACS [7,8].

The outer membrane of Gram-negative bacteria consists of lipopolysaccharides (LPS) *aka* endotoxins. The elevated level of endotoxin in systemic circulation is associated with a threefold increased risk of developing atherosclerosis [9]. Endotoxins bind Toll-Like Receptor-4 (TLR-4) present on the intestinal, immune and endothelial cells, and form a TLR-4 receptor cluster with the Cluster of Differentiation-14 (CD14). TLR4 polymorphism confers reduced risk of atherosclerosis [10], while CD14 promoter polymorphism leads to an increased risk of ACS and atheromatous plaque vulnerability [11]. The possible mechanism include endotoxin-induced damage to the endothelial cells which stimulates macrophage transformation into foam cells promoting atherosclerosis [12]. Studies from our lab have shown that endotoxin – at clinically achievable concentration cause an increase in intestinal

permeability both *in vitro* and *in vivo* models [13]. The increase in serum endotoxin levels causes a further increase in intestinal permeability which in turn allows surge of endotoxins, culminating in an amplification cascade of increase in intestinal permeability and worsening endotoxemia.

Very few studies have described the link between gut-microbiome and atherosclerosis or ACS. In one such interesting study, Proteobacteria were found within the endarterectomy samples of atherosclerotic plaques [14] and further presence of Proteobacteria in blood was associated with an increased risk of cardiac events (Odds ratio 1.56, 95% CI 1.12–2.15) [15]. A well described link between the intestinal microbiome and ACS is the metabolite trimethylamine-N-Oxide (TMAO). The dietary phospholipids, such as phosphatidylcholine and L-carnitine are metabolized by intestinal bacteria to yield trimethylamine (TMA) that is subsequently absorbed and metabolized into TMAO in the liver [16]. Multiple studies have revealed an association between TMAO and risk for cardiovascular events [16,17].

Proteobacteria is a primary source of TMAO and endotoxemia, which are risk factors for atherosclerosis and future ACS events [18]. Therefore, we hypothesized that ACS patients have leaky-gut condition characterized by an increased Proteobacteria (dysbiosis), which leads to higher levels of blood TMAO and LPS. Goal of our study was to investigate (immediately after an acute cardiac event) whether patients with ACS have underlying dysbiosis, increased intestinal permeability, and higher levels of serum endotoxins and TMAO.

## 2. Methods

### 2.1. Study design overview

This is a pilot case-control study performed in a single tertiary center; University of New Mexico Hospital, between March 2014 and November 2016. For detailed materials and methods please refer to Supplementary methods section.

### 2.2. Inclusion criteria

Patients admitted with a diagnosis of ACS. The definition of ACS as described by the American Heart Association was used, including ST-elevation myocardial infarction (STEMI) and non-ST segment elevation myocardial infarction (NSTEMI). All patients were recruited in the study within 72 h of admission. Healthy controls (matched to cases by age and sex) met the following criteria: have normal body mass index ( $18.5 < \text{BMI} < 24.9$ ), no hypertension, diabetes or dyslipidemia, no prior heart attacks or strokes, no tobacco use, and no antibiotic use within three months prior to enrollment. Controls were recruited through the University of New Mexico Clinical and Translational Science Center (CTSC) registry of healthy controls.

### 2.3. Exclusion criteria

Patients less than 19 years, pregnancy, abnormal kidney function, heart failure, intensive care unit (ICU) level of care.

#### 2.4. Sample size

was calculated to achieve a statistical power >80%, and a level of significance  $\alpha = 0.05$ . To detect a 100% increase in intestinal permeability in the ACS group compared to the control group; 17 patients per groups were required (effect size based on prior work in our lab). Therefore, we enrolled 19 subjects in each healthy and control group.

#### 2.5. Measuring barrier functions

Intestinal permeability was measured using mannitol (M) and lactulose (L) urinary excretion method. L/M ratio is a well-validated marker for intestinal permeability [19]. The concentration of mannitol and lactulose present in the urine was determined by an enzyme-based assay (Megazyme, Ireland) and quantified by spectrophotometry. The lactulose-to-mannitol ratio (L/M ratio) was calculated by dividing the fractional (percentage) of urinary excretion of lactulose over the fractional excretion of mannitol.

#### 2.6. Quantifying serum TMAO levels

Serum TMAO was measured using a highly sensitive liquid chromatography electrospray ionization mass spectrometry (LCMS), performed at Cleveland HeartLab, Inc. (Cleveland, OH, USA).

#### 2.7. Determining serum LPS levels

LPS serum level was measured using a Limulus ameocyte lysate (LAL) chromogenic endotoxin quantification kit (Thermo Scientific, IL, USA).

#### 2.8. DNA extraction

DNA extraction from stool samples was performed using the ZR Fungal/Bacterial Miniprep Kit (Zymo Research, CA, USA).

#### 2.9. MetaVx™ mammalian library preparation and Illumina MiSeq sequencing

Next generation sequencing library preparations and Illumina MiSeq sequencing were conducted at GENEWIZ, LLC. (South Plainfield, NJ, USA). Please refer to Supplementary methods section.

#### 2.10. Sequence analysis by QIIME pipeline

Please refer to Supplementary methods section.

#### 2.11. Predicting functional content from 16S gene

Please refer to Supplementary methods section.

#### 2.12. Statistical tests

Standard unpaired *t*-test (two-tailed) was used for data with normal distribution, and non-parametric *t*-test (Satterthwaite and Mann-Whitney-Wilcoxon) were used for data with unequal variance, or data that are not normally distributed.

### 3. Results

#### 3.1. Subject characteristics

19 ACS patients and 19 healthy controls were recruited. More than two thirds of ACS patients as well as healthy controls were Caucasian (non-Hispanic Whites) males, patients were in their mid-fifties as expected for the first ACS episode, and were slightly older than the healthy controls. ACS patients were overweight (mean BMI of 28.3); a 4-point higher BMI than healthy controls. All ACS patients underwent urgent coronary angiography; eighty-nine percent had STEMI. The mean troponin level was markedly elevated at 30.07 ng/mL, while 63% patients had a single vessel disease. Complete characteristics of patients and healthy controls are described in Table 1.

#### 3.2. Increased intestinal permeability in ACS patients

The absorption of mannitol and lactulose are used to assess overall mucosal absorptive surface area and also paracellular permeability, respectively, such that mucosal cell damage and reduced surface area are associated with decreased mannitol absorption while increased intestinal tight-junctional permeability (leaky gut) correlates with an increased lactulose absorption. The lactulose-to-mannitol ratio (L/M ratio) was calculated by dividing the fractional (percentage) of urinary excretion of lactulose over the fractional excretion of mannitol. Fifty percent of patients (n = 8) and most controls (n = 16) completed the L/M permeability test. ACS patients had leaky gut as evidenced by the L/M ratio that was three times higher than the healthy controls (P = 0.014) (Fig. 1A and B).

#### 3.3. Healthy and ACS subjects had similar blood TMAO and LPS levels

We expected ACS group to show significantly higher levels of TMAO but surprisingly, all subjects had similar serum TMAO concentrations. Mean TMAO levels for ACS and healthy groups were  $5.8 \mu\text{M} \pm 4.8$  vs  $5 \pm 2.9$  (P = 0.5) respectively, (Fig. 1C). Further, when the mean serum LPS level was measured by LAL chromogenic assay, there was a trend towards a higher LPS levels in the ACS group but not statistical significant (P = 0.3) (Fig. 1D).

#### 3.4. Microbiome analysis overview

**Alpha Diversity:** We sequenced all samples to detect all available OTUs as shown in the observed-OTUs rarefaction plot (Fig. 2A). In both groups the number of observed OTUs (mean  $\pm$ SD) was more than 1000 ( $1928.9 \pm 1165.1$  vs.  $1336.2 \pm 611.7$ , P-value = 0.19). **Phylogenetic Diversity (PD):** is an indicator of biodiversity, PD was measured for all samples (Fig. 2B). Our results showed no significant difference in PD between the two groups: mean PD ( $\pm$ SD) in ACS patients vs healthy controls was  $111.63 \pm 45.65$  vs.  $86.55 \pm 25.69$ , P-value = 0.16. **Chao1 Index:** a measure of sample richness was performed for all subjects (Fig. 2C). The Chao1 index was similar in ACS patients and healthy controls: mean Chao1 index was  $3303.3 \pm 1747.6$  vs.  $2415.8 \pm 977.7$ , P-value = 0.2 (Fig. 2D). **Beta diversity:** Unique Fraction Distance (Unifrac) distance (weighted for abundance) was uniform; a mean distance of  $\sim 0.4$  was noted between samples within the same group and between samples within different groups (Fig. 2E). **Principal Component Analysis (PCoA):** of Unifrac

distance showed similar distribution of ACS and healthy subjects along the three PCA axes; no distinct group clustering was observed (Fig. 2F).

### 3.5. Major bacterial phyla in both groups

16S rRNA analysis revealed the relative abundance of intestinal major bacterial phyla in both groups (Fig. 3A) and in individual subjects (Fig. 3B). Both groups had Bacteroidetes and Firmicutes as the two major phyla, and a small percentage of Proteobacteria and Actinobacteria. Although the mean Proteobacteria percentage was double in ACS patients compared to controls ( $4.1 \pm 3.8$  vs  $2.1 \pm 1.7$ ), the result was not statistically significant ( $P$ -value = 0.056) (Fig. 3C). There was also no significant difference in Bacteroidetes, Firmicutes, and Actinobacteria relative abundance between the two groups (Fig. 3(D–F)).

### 3.6. Dysbiosis with increased relative abundance of Gammaproteobacteria in ACS

Lower taxonomical levels in Phylum Proteobacteria reveals the relative abundances of Gammaproteobacteria (Class) and Enterobacteriaceae (Family) were higher in ACS patients compared to controls. Relative abundance values were statistically significant at the Gammaproteobacteria level (Fig. 4(A–C)). Relative abundance of different bacterial OTUs was calculated by group and in individual subjects (Fig. 4D and E). More than twenty bacterial genera/families had more than 2-fold difference between the two groups (Fig. 4F). ACS patients had higher levels of Prevotella and controls had higher overall Bacteroidales levels. Elevated Prevotella/Bacteroides (B/P ratio) is a microbiome enterotype linked to carbohydrate rich diet and cardiovascular risk such as hypertension (HTN) [20].

### 3.7. Functional analysis revealed microbiome source contributing LPS biosynthesis markedly increased in ACS patients

A predictive functional analysis of LPS biosynthesis showed a marked difference in the phyla contributing LPS biosynthesis. In ACS patients, the main source of LPS biosynthesis pathways was Proteobacteria, compared to Bacteroidetes in the healthy controls group (Fig. 4G). 16S rRNA sequencing analysis showed no increased abundance of any specific taxon capable of producing LPS.

All major results for both groups are summarized in Supplementary Table S1.

## 4. Discussion

We found that ACS patients have increased intestinal tight-junctional permeability (leaky gut); the lactulose-to-mannitol urinary excretion ratio was three times higher in patients compared to controls;  $0.06 \pm 0.07$  vs  $0.02 \pm 0.02$  ( $P = 0.014$ ). There was no significant difference in the levels of TMAO and LPS between the two groups. However, functional analysis revealed that LPS biosynthetic pathway in ACS patients is mainly comprised of Proteobacteria as opposed to Bacteroidetes in healthy controls. (Fig. 4G).

Additionally, ACS patients have nine-fold increase in intestinal Gammaproteobacteria (a Class that includes opportunistic pathogenic Gram-negative bacteria), compared to healthy controls. Proteobacteria level was 2-folds higher in ACS patients, with no statistical

significance ( $P = 0.056$ ). The Proteobacteria (facultative anaerobes) level detected in stool samples (luminal fraction) is expected to be lower than what is actually present on the mucosal surface of distal intestine (mucosa-associated fraction) [21], suggesting that ACS patient's mucosal surface might have significantly higher relative abundance of Proteobacteria. ACS patient fecal samples clearly suggest some degree of dysbiosis compared to healthy controls, following an acute cardiac event.

Intestinal Proteobacteria is the most unstable of the four-major intestinal bacterial phyla [Bacteroidetes, Firmicutes, Actinobacteria and Proteobacteria] [22]. High levels of Proteobacteria is a key feature of dysbiosis in many inflammatory and metabolic conditions such as metabolic syndrome, obesity, diabetes mellitus and inflammatory bowel disease [23,24]. A recent study on atherosclerotic cardiovascular disease (218 patients) showed increased Enterobacteriaceae (a major Family within the Class Gammaproteobacteria) compared to controls [25].

Previous studies have confirmed a strong correlations between dysbiosis with higher levels of endotoxins/TMAO and development of a future cardiovascular event, but such studies are scarce for patients during acute cardiac events. With the limited number of ACS subject's sample analysis, we showed an increase in Gammaproteobacteria in ACS patients and an increase in intestinal permeability, however, increased LPS levels were not observed.

Gram-negative bacteria produce different endotoxins—with major variation in the immunogenic Lipid A portion—that bind to TLR4. Endotoxins (with different lipid A) activate innate immune system and trigger a largely variable immunogenic response [26–28] – leading to a range of disease conditions. For example, LPS from Proteobacteria is structurally distinct from Bacteroides LPS, and Proteobacteria LPS is markedly more immunogenic [26,27]. Although LPS levels were not different between the two groups in this study, it is possible that ACS patients have higher immunogenic Proteobacteria-LPS based on our functional analysis, this can only be confirmed with analyzing different types of serum LPS, which is beyond the scope of present study.

We found no difference in TMAO levels between the two groups, this result is consistent with a recent study in patients with acute stroke and Transient Ischemic Attacks (TIAs) [29]; wherein patients had lower levels of TMAO compared to controls. This doesn't contradict the previous large studies where high levels of TMAO correlated with increased risk of future acute cardiac events. It is likely that TMAO level is associated with a long-term risk of cardiovascular events and level at the time of the ACS event can be normal or low. In addition, TMAO levels are known to fluctuate widely over the course of time ( $R^2 = 0.29$ ,  $P = 0.003$ ) [30].

## 5. Limitations

Only 50% of ACS patients underwent the intestinal permeability test due to delayed IRB approval, leading to a smaller than planned sample size. Intestinal permeability was three times higher in ACS patients compared to controls (large effect size) and therefore result was statistically significant. ACS patients had higher BMI compared to healthy controls

(mean BMI of ACS patients is  $28.31 \pm 1.9$  vs  $24.3 \pm 0.39$  for healthy controls). Elevated BMI can alter the intestinal microbiome and affect the study results, we reanalyzed the study endpoints including ACS patients who have normal BMI (BMI less than 25) compared to healthy controls. Our results still show difference between the two groups after we correct for the BMI variable (Supplementary Table S2).

## 6. Future directions

To establish a clear association among ACS, leaky-gut and gut microbiota, a larger scale exploratory study is required. Recruiting a significantly larger number of patients to investigate detailed changes at the bacterial strain level (which we propose doing using a full metagenomics and metatranscriptomics approach) will add greater insight on the potential function of the microbiome and interactions with the host. Outcome of such studies will open the door for interventional studies targeting the microbiome composition and function using probiotics and prebiotics to alter enzymatic pathways affecting TMAO metabolism, tightening the intestinal TJ, and balancing the microbiome composition.

## 7. Conclusions

Dysbiosis affects patients with ACS, with an increase in intestinal permeability. It is unclear if dysbiosis and leaky gut are mere associations, or key contributing factors in acute cardiac events hence, large scale exploratory studies are required to find the exact role of dysbiosis in ACS patients.

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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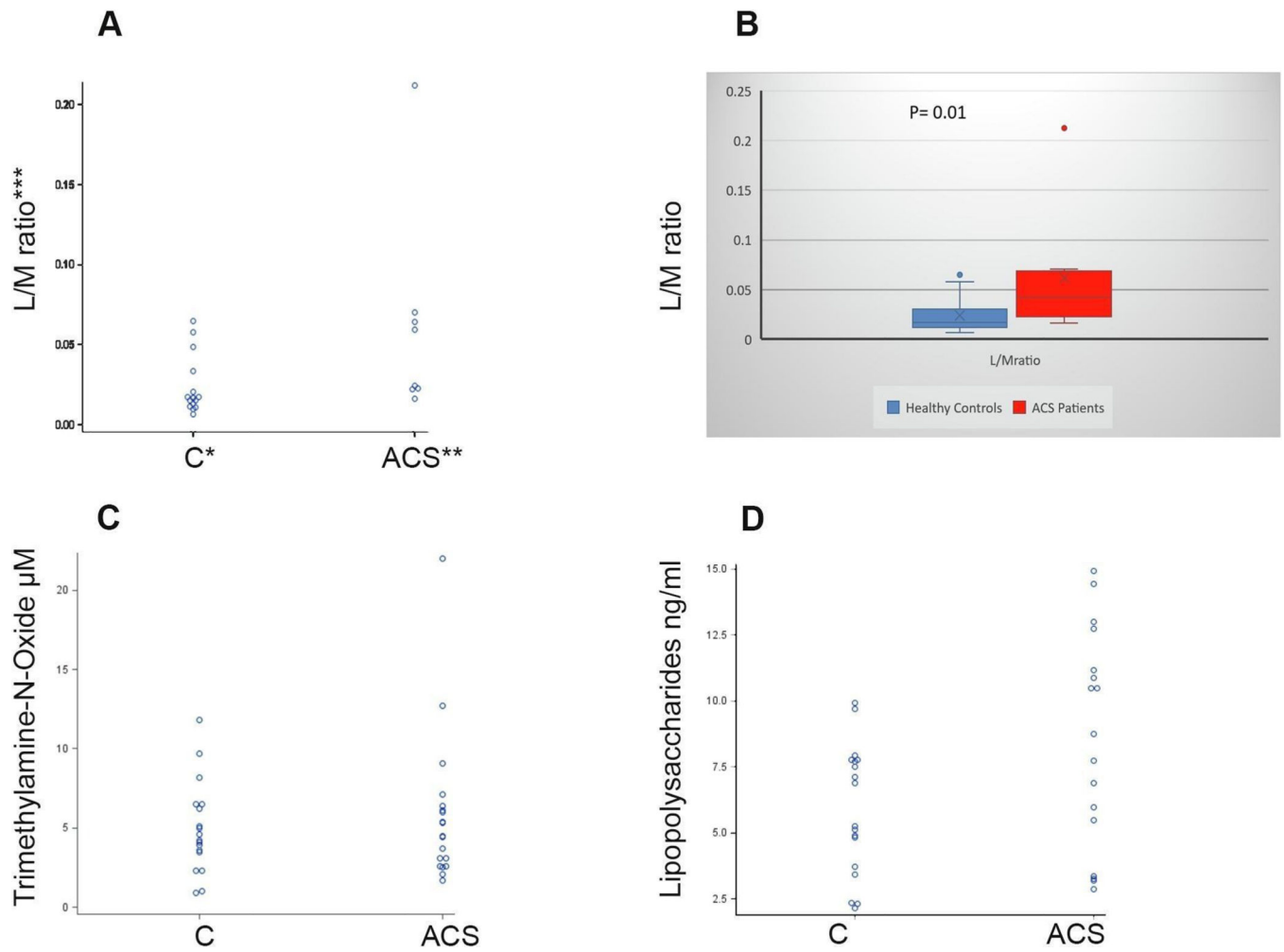
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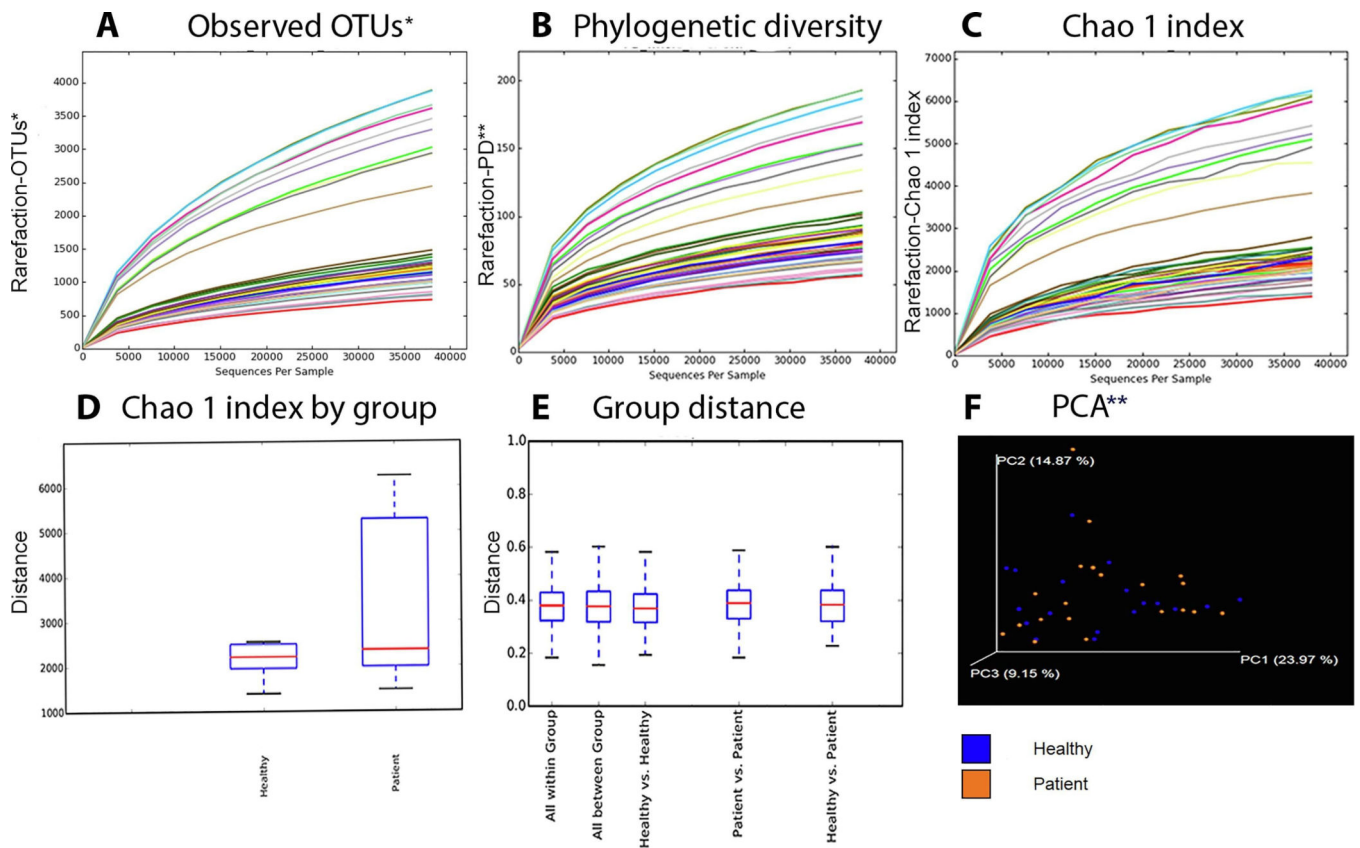


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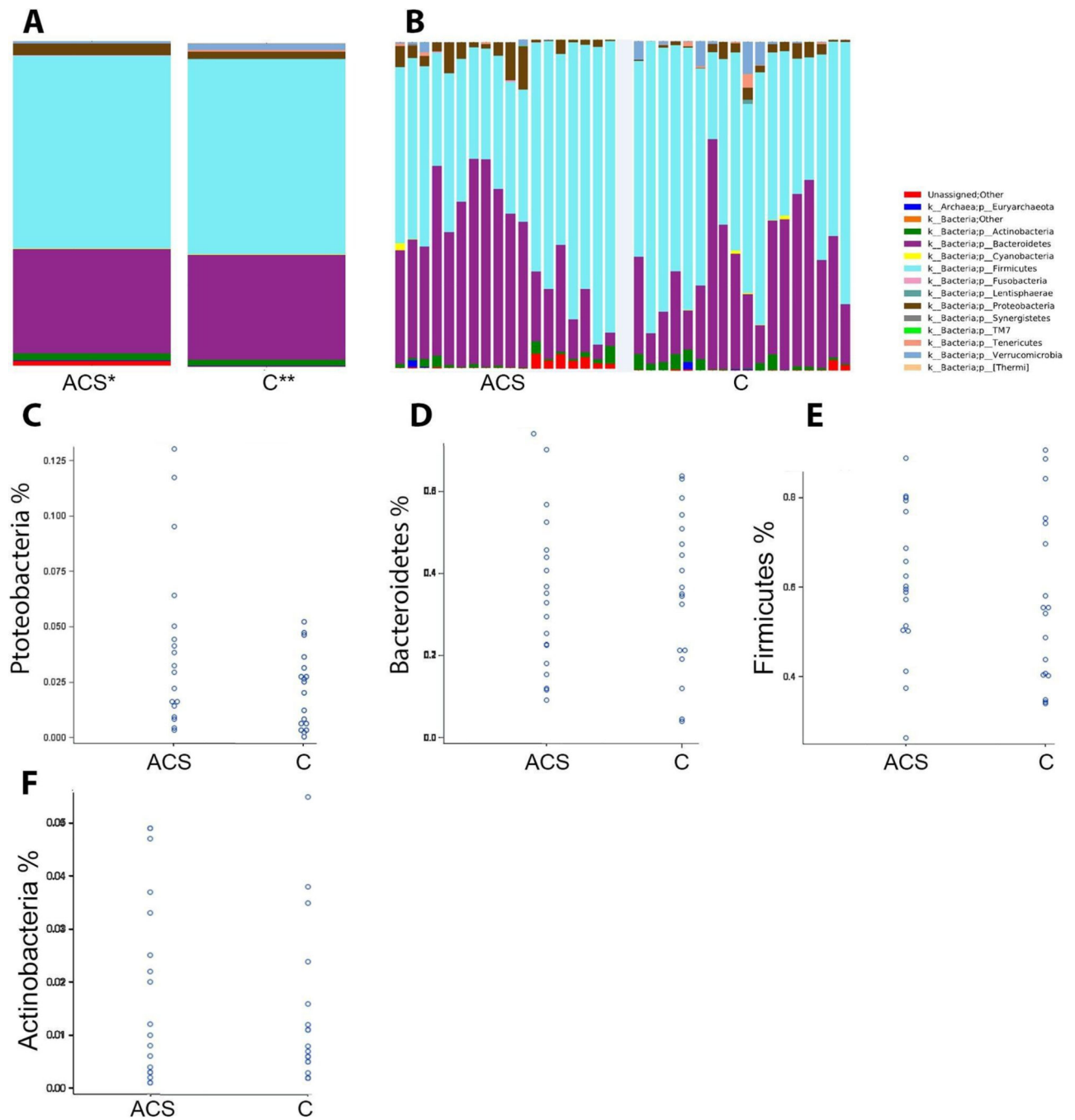
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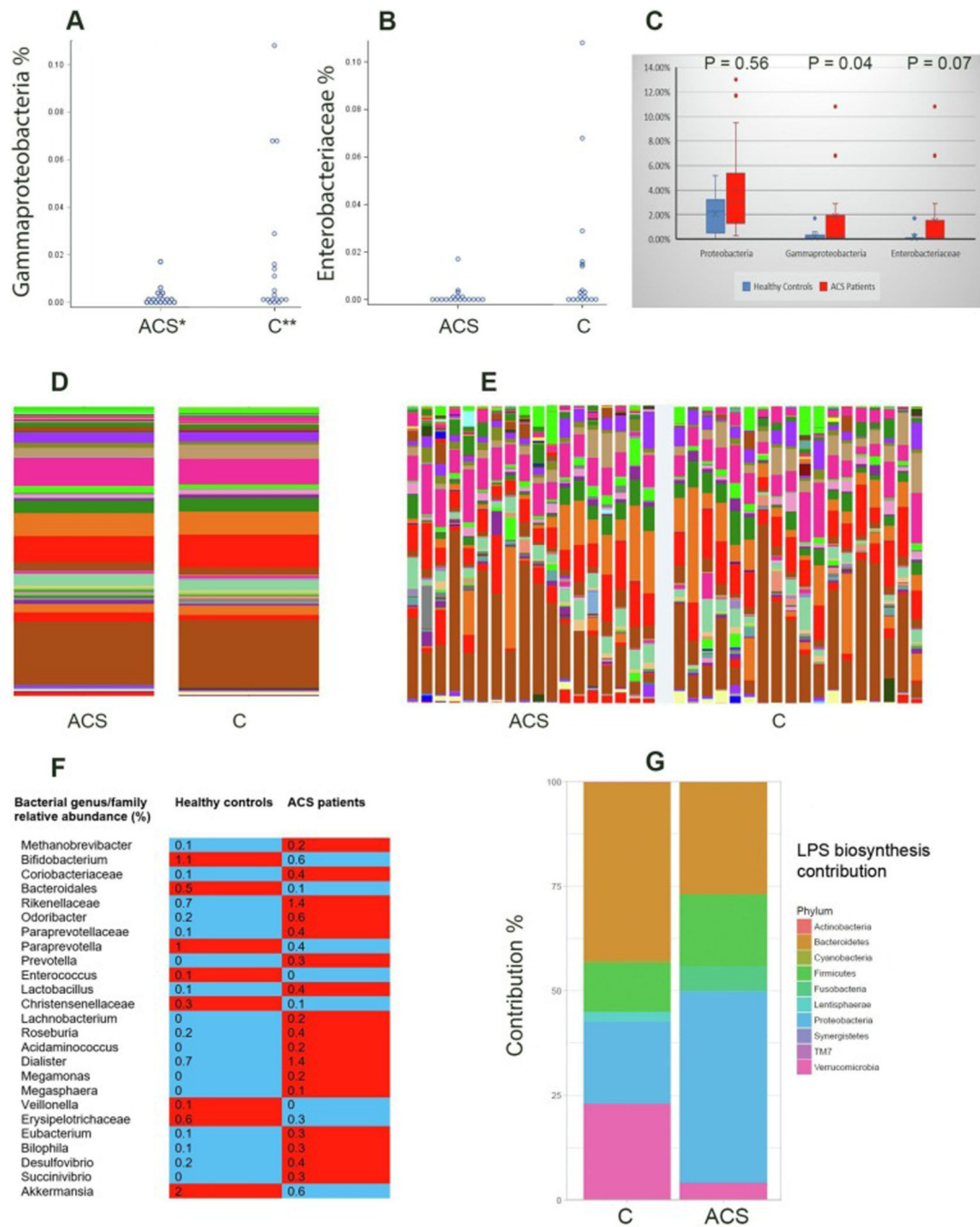
**Fig. 1.** Lactulose to mannitol (L/M) ratio, trimethylamineoxide (TMAO) and lipopolysaccharide (LPS) levels. (A–B) L/M ratio in ACS patients and healthy controls, and mean L/M ratio in both groups. (C) TMAO serum levels in ACS patients and healthy controls. (D) LPS serum levels in ACS patients compared to healthy controls. \*Healthy controls. \*\*Acute coronary syndrome. \*\*\*Lactulose to mannitol ratio.



**Fig. 2.** Diversity of the intestinal microbiome in ACS patients and healthy controls. (A–C) Rarefaction plots showing number of OTUs, phylogenetic diversity and Chao1 index for individual subjects. (D) Chao1 index per group. (E) Weighted unique fraction group distances. (F) Principal component analysis of weighted unique fraction distance. \*Operational taxonomic unit. \*\* Principal component analysis.



**Fig. 3.** Relative abundance of major bacterial phyla in ACS patients and healthy controls. (A) Relative abundance of bacterial phyla per group. (B) Relative abundance of bacterial phyla per subject. (C–F) Levels of fecal Proteobacteria, Bacteroidetes, Firmicutes, and Actinobacteria in ACS patients compared to healthy controls. \*Acute coronary syndrome. \*\*Healthy controls.



**Fig. 4.** Relative abundance of Gammaproteobacteria, Enterobacteriaceae and other bacterial genera in study subjects. (A–B) Levels of fecal Gammaproteobacteria and Enterobacteriaceae in ACS patients compared to healthy controls. (C) Summary of Proteobacteria, Gammaproteobacteria and Enterobacteriaceae levels per group. (D, E) Relative abundance of bacteria per group and per subject at the genus level, for legend refer to Supplementary Fig. 1 (S1 Figure). (F) Bacterial genera with more than 2-fold difference between the two groups. Red color indicates higher abundance. (G) Bacterial phyla contribution to LPS

synthesis in ACS patients and healthy controls. \*Acute coronary syndrome. \*\*Healthy controls.

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**Table 1**

Baseline characteristics of ACS patients and healthy controls.

Baseline characteristics	ACS group (n =19)	Control group (n =19)	P-value
Age (mean $\pm$ SD <sup>*</sup> )	54.4 $\pm$ 2.2	49 $\pm$ 1.6	0.07
Gender, Male (n, %)	14 (74)	13 (68)	0.7
BMI (kg/m <sup>2</sup> ) (mean $\pm$ SD)	28.31 $\pm$ 1.9	24.3 $\pm$ 0.39	0.004
Race			
Caucasian	13 (68)	14 (74)	0.72
Others	6 (32)	5 (26)	
<b>Clinical characteristics</b>			
	<b>n (%)</b>	<b>n (%)</b>	
Alcohol use	7 (37)	0 (0)	-
<i>Tobacco use</i>			
Current smoker	4 (21)	0 (0)	-
Ex-smoker	4 (21)	0 (0)	-
Non-smoker	11 (58)	0 (0)	-
<i>Past Medical History</i>			
Family history of CAD <sup>**</sup>	13 (68)	0 (0)	-
Diabetes Mellitus Type II	4 (21)	0 (0)	-
Hypertension	6 (32)	0 (0)	-
Prior ACS <sup>‡</sup>	2 (10)	0 (0)	-
Admission diagnoses		NA	NA
STEMI <sup>§</sup>	17 (89)		
NSTEMI <sup>  </sup>	2 (10)		
<i>Intervention</i>			
Coronary angiography with PCI <sup>¶</sup>	19 (100)		
Stent placement	18 (95)		
<i>Number of affected vessels</i>			
One-vessel disease	12 (63)		
Two-vessel disease	6 (32)		
Three-vessel disease	1 (5)		
Mean Troponin level	30.07 ng/mL		

\* Standard deviation.

\*\* Coronary artery disease.

‡ Acute coronary syndrome.

§ ST-segment elevation myocardial infarction

|| Non-ST segment myocardial infarction.

¶ Percutaneous coronary intervention.