High-throughput sequencing identifies distinct fecal and mucosal gut microbiota correlating with different mucosal proteins

Li-na Dong [1] , **Jun-ping Wang** Corresp., [2] , **Ping Liu** [3] , **Yun-feng Yang** [2] , **Jing Feng** [2] , **Yi Han** [2]

[1] Central Laboratory, Shanxi Provincial People's Hospital, Affiliate of Shanxi Medical University, Taiyuan, China
[2] Department of Gastroenterology, Shanxi Provincial People's Hospital, Affiliate of Shanxi Medical University, Taiyuan, China
[3] Department of Gynaecology, Shanxi Provincial People's Hospital, Affiliate of Shanxi Medical University, Taiyuan, China

Corresponding Author: Jun-ping Wang
Email address: wangjp8396@sohu.com

The intestinal microbiota is associated with human health. The luminal microbiota (LM) and mucosa-associated microbiota (MAM) are distinct ecosystems with different metabolic and immunological functions. Several studies have examined the correlations between the gut microbiota and clinical indices, but few have investigated the relationships between the microbiota and mucosal proteins. We characterized the intestinal LM and MAM in Chinese people and examined the association between these communities and the expression of mucosal proteins. Fresh fecal samples and distal colonic mucosal biopsies were collected from 32 subjects before (fecal) and during (mucosal) flexible sigmoidoscopy. We used high-throughput sequencing targeting the 16SrRNA gene V3–V4 region to analyze the samples and reverse transcription(RT)–PCR to detect the expression of colonic proteins BDNF, ZO1, TLR2, TLR4, AQP3, and AQP8. Differences in the stool and mucosal microbiota were identified and a correlation network analysis performed. The LM and MAM populations differed significantly. In LM, the microbiota composition correlated significantly positively with host age, and Firmicutes (phylum) correlated positively with body mass index (BMI), but inversely with ZO1.At the genus level, systemic indices, such as age, BMI, and BDNF, correlated predominantly with LM, whereas systemic and local indices, such as TLR2, correlated with both MAM and LM. ZO1 and TLR4 which usually exert a local effect, mainly correlated with MAM. Different bacteria were associated with the expression of different proteins. Our data suggest that The microbial compositions of LM and MAM differed. Different gut bacteria may play different roles by regulating the expression of different proteins.

PeerJ Preprints | https://doi.org/10.7287/peerj.preprints.2526v1 | CC BY 4.0 Open Access | rec: 16 Oct 2016, publ: 16 Oct 2016

High-throughput Sequencing Identifies Distinct Fecal and Mucosal Gut Microbiota Correlating with Different Mucosal Proteins

Li-na Dong et al. Different Fecal and Mucosal Microbiota.

Li-na Dong[1], Jun-ping Wang[2]*, Ping Liu[3], Yun-feng Yang[2], Jing Feng[2], Yi Han[2]

*Corresponding author:

Jun-ping Wang. Tel: +86-0351-4960141;E-mail: wangjp8396@sohu.com

Name	Highest academic degree	Email	Postal addresses
Li-na Dong	MSc	donglina1983@126.com	Shanxi Provincial People's Hospital, 29 Shuangta Road, Taiyuan 030012, China
Jun-ping Wang	PhD	wangjp8396@sohu.com	
Ping Liu	MSc	wangjp8396@126.com	
Yun-feng Yang	MSc	yangyunfeng024@126.com	
Jing Feng	MSc	fengjing791120@126.com	
Yi Han	MSc	dzw1008@163.com	

[1]Central Laboratory, Shanxi Provincial People's Hospital, Affiliate of Shanxi Medical University, Taiyuan, China.

PeerJ Preprints | https://doi.org/10.7287/peerj.preprints.2526v1 | CC BY 4.0 Open Access | rec: 16 Oct 2016, publ: 16 Oct 2016

[2]Department of Gastroenterology, Shanxi Provincial People's Hospital, Affiliate of Shanxi Medical University, Taiyuan, China

[3]Department of Gynaecology, Shanxi Provincial People's Hospital, Affiliate of Shanxi Medical University, Taiyuan, China

Author contribution: Li-na Dong performed the majority of experiments, analyzed the data and drafted the manuscript ; Jun-ping Wang, Ping Liu participated in the design of this study and manuscript reviewer, Yun-feng Yang, Jing Feng, Yi Han performed specimen collection. All authors read and approved the final manuscript.

Supported by the International Science and Technology Cooperation Project of Shanxi （No.2013081066), Science Foundation of Health and family planning commission of Shanxi Province(No.201201059).The funding bodies had no role in the study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Institutional review board statement: The study was approved by the Ethics Committee of Shanxi People's Hospital.

Conflict-of-interest statement: The authors declare that they have no competing interests.

Data sharing statement: No additional data are available.

Correspondenceto: Wang Jun-ping, Professor, Department of Gastroenterology, Shanxi Provincial People's Hospital, Affiliate of Shanxi Medical University, Shanxi Provincial People,s Hospital, 29 Shuangta Road, Taiyuan 030012, China.

E-mail:wangjp8396@sohu.com

Tel: +86-0351-4960141

ABSTRACT

The intestinal microbiota is associated with human health. The luminal microbiota (LM) and mucosa-associated microbiota (MAM) are distinct ecosystems with different metabolic and immunological functions. Several studies have examined the correlations between the gut microbiota and clinical indices, but few have investigated the relationships between the microbiota and mucosal proteins.We characterized the intestinal LM and MAM in Chinese people and examined the association between these communities and the expression of mucosal proteins. Fresh fecal samples and distal colonic mucosal biopsies were collected from 32 subjects before (fecal) and during (mucosal) flexible sigmoidoscopy. We used high-throughput sequencing targeting the 16SrRNA gene V3－V4 region to analyze the samples and reverse transcription(RT)－PCR to detect the expression of colonic proteins BDNF, ZO1, TLR2, TLR4, AQP3, and AQP8. Differences in the stool and mucosal microbiota were identified and a correlation network analysis performed. The LM and MAM populations differed significantly. In LM, the microbiota composition correlated significantly positively with host age, and Firmicutes (phylum) correlated positively with body mass index (BMI), but inversely with ZO1.At the genus level, systemic indices, such as age, BMI, and BDNF, correlated predominantly with LM, whereas systemic and local indices, such as TLR2, correlated with both MAM and LM. ZO1 and TLR4 which usually exert a local effect, mainly correlated with MAM. Different bacteria were associated with the expression of different proteins. Our data suggest that The microbial compositions of LM and MAM differed. Different gut bacteria may play different roles by regulating the expression of different proteins.

Keywords Gastrointestinal microbiota·high-throughput sequencing·16S rRNA gene Mucosal Proteins

Introduction

The intestinal microbiota is a complex community of Bacteria, Archaea, viruses, and Eukarya. A wide variety of bacterial species in the gastrointestinal tract exert numerous effects on the host and influence a variety of gastrointestinal functions[1]. Fecal samples (representing the luminal niche) are examined in most studies ofthe intestinal microbiota because they are easily collected. However, recent researchhas shown that the microbial compositions ofthe luminal microbiota (LM) and the mucosa-associated microbiota (MAM) differ, suggesting that these two distinct microbial populations play different roles within the intestinal microbiota ecosystem [2]. LM is the microbiota involved in the whole intestine, whereas MAM represents a special niche. Because MAM is in close contact with the host, it may play a more prominent role in the intestinal barrier function, whereas LM may play a key role in metabolic activities and nutrient harvest[3]. However, the different functions ofLM and MAM are unknown.

Many proteins are involved in the colonic microbe–host interactions. The tight junction proteins constitute a critical platform that regulates the integrity of the epithelial barrier and maintains the activation of the mucosal immunitywithin an acceptable range [4].The effects of brain-derived neurotrophic factor (BDNF) in the gut are beginning to be identified; there is growing evidence that BDNF also plays an important role in gastrointestinal functions. Wang et al. found that the activation of PAR-2 signaling by fecal supernatants from patients withirritable bowel syndrome (IBS) with diarrhea promoted the expression of colonic BDNF, thereby contributing to IBS-like visceral hypersensitivity [5]. Toll-like receptors (TLRs) are pattern recognition receptors expressed by various cells in the gastrointestinal tract. The microbiota may directly interact with the TLRs and regulate the gut immune responses, especially through the activation of TLRs [6].

Water transport through the human digestive system is physiologically crucial for maintaining the water homeostasis of the body and ensuring digestive and absorptive equilibria. Aquaporins (AQPs) are important transmembrane water channel proteins. Guttman et al. foundthat the altered localization of AQPs was partly dependent on the bacterial type III effector proteins EspF and EspG[7].

We speculate that different bacteria play different roles in the colon, and that different bacteria regulate the expression of different proteins,thus affecting intestinal functions. However, few data are available on the correlation between the intestinal microbiota and mucosa-associated proteins.

In this study, we used high-throughput pyrosequencing of the bacterial 16S rRNA gene to compare the microbial communities in the fecesand mucosa of Chinese subjects, and to study their association with the expression of colonic mucosal proteins (ZO1, BDNF, TLR2, TLR4, AQP3, and AQP8) and the clinical features (age and body mass index [BMI]) of the host.

Materials and Methods

Study Subjects

Thirty-two Chinese patients were recruited from the Department of Gastroenterology, Shanxi Provincial People’s Hospital,in 2013 and 2014. None of the subjects enrolled in the study had taken corticosteroids, opioids, or antibiotics in the 6 months preceding the study; none had any systemic comorbidity;andnone had a history of excessive alcohol intake (>20 alcoholic drinks per week). Patients with a prior history of gastrointestinal surgery or intestinal organic disease were excluded. All subjects gave their signed informed consent before participation. The study was performed in accordance with the principles of the Declaration of Helsinki, and the study protocol was approved by the Ethics Committee of Shanxi Provincial People's Hospital, China.

Stool Sample Processing and DNA Extraction

The fecal samples were collected at home <12 h before colonoscopy, frozen immediately at－20 °C, and transported within 12 h to the study center, where they were stored at－80 °C until analysis. Bacterial DNA was extracted from the fecal samples withthe QIAamp® DNA Stool Mini Kit (Qiagen, Hilden, Germany), according to the manufacturer's protocol. The DNA concentrations were quantified with an Eppendorf BioSpectrometer® (Eppendorf,Hamburg, Germany).

Genomic DNA Extraction

Total genomic DNA was extracted from samples of digests from the colon with a QIAamp DNA Mini Kit (Qiagen), according to the manufacturer's instructions. The concentration and purity of the genomic DNA were measured with an Eppendorf BioSpectrometer.

PCR Amplification of V3–V4 Region of Bacterial 16S rRNA Gene and Illumina Sequencing

The bacterial genomic DNA was used as the template to amplify the V3–V4 hypervariable region of the 16SrRNA gene with the forward primer (5′-GACTACHVGGGTATCTAATCC-3′)and the reverse primer (5′-CCTACGGGNGGCWGCAG-3′).

Bioinformatic Analysis

Pairs of reads from the original DNA fragments were merged using FLASH, which was designed to merge pairs of reads when the original DNA fragments were shorter than twice the read length. The sequencing reads were assigned to each sample according to a unique barcode and were analyzed with the QIIME (Quantitative Insights Into Microbial Ecology) software package and the UPARSE pipeline. In brief, the reads were filtered with the QIIME quality filters using the default settings for Illumina processing, and the operational taxonomic units (OTUs) were selected using the UPARSE pipeline. The samples were sequenced on an Illumina MiSeq Benchtop Sequencer and the bioinformatic analysis were performed by Genesky Biotechnologies Inc., Shanghai, China.

The sizes of the bacterial groups were expressed as percentages of the total bacteria.

Quantitative Real-time Polymerase Chain Reaction (qPCR)

The total mucosal RNAs were extracted from the colonic biopsies using the TaKaRa MiniBEST Universal RNA Extraction Kit (TaKaRa), according to the manufacturer's instructions. The mRNA concentrations and purity were measured with an Eppendorf BioSpectrometer. After reverse transcription with PrimeScript Reverse Transcriptase Mix (TaKaRa), which converted the total RNA to cDNA, the expression of the *BNNF*, *ZO1*, *TLR2*, *TLR4*, *AQP3*, and *AQP8*genes was determined with qPCR and SYBR Green technology on a Bio-Rad CFX96™ Q-PCR instrument(Bio-Rad, USA) for each sample in duplicate. The specific primers are listed in Table1. Each amplification reaction was run in duplicate in a final volume of 20μl containing 10μl of Power SYBR Green PCR master mix, 400 nmol of the forward and reverse primers, and 1μl of cDNA. All the qPCRs were optimized and performed in 0.2ml 96-well plates,with the following cycling program: initial denaturation at 95 °C for 10 min, followed by 40 cycles of 95 °C for 15 s and 60 °C for 30s. Fluorescence was measured at the last step of each cycle. To determine the specificity of the amplification, the dissociation characteristics of the double-stranded DNA were determined with a melting curve analysis. The dissociation of the PCR products was monitored by slowly heating them,in increments of 0.1 °C/s, from 55 °C to 95 °C, with fluorescence measurements made at 0.1 °C intervals. The correct PCR product length was confirmed with gel electrophoresis. Negative controls lacking the template DNA were included in triplicate. Standard curves for the target bacterial groups were generated using serial dilutions (corresponding to approximately 10^1–10^{10}copies/μl) of the purified and quantified PCR products generated from genomic DNA with standard PCR. The mRNA levelswere absolutely quantified by converting the sample cycle threshold (Cq) values to concentrations (copies per μl) based on the standard curves [8].

Statistical Analysis

All statistical analyses were performed with SPSS 22.0 for Windows (SPSS Inc., USA).To determine the statistical differences between the two groups, we usedan independent-samples*t*test and the Mann–Whitney test. Correlations were determined with Spearman's correlation. The resulting pvalues were adjusted using the Benjamini–Hochberg false discovery rate (FDR) correction. Only FDR-corrected p values below 0.05 were considered significant.

Results

Study Population

We investigated 64 samples from 32 subjects. All subjects provided both a fecal sample and a colonic mucosal sample. The study population consisted of 50% females,and had a mean age of 49 (20–65) years and a mean BMI of 23.24.

Characteristics of the Pyrosequencing Results

We obtained a total of 4,351,929 raw reads and 3,545,053 reads remained after filtering. The sequencing analysis of the 64 samples identified 1026 OTUs. The rarefaction curves tended to approach the saturation plateau, indicating that the number of samples used in this study was reasonable. The same tendency was found in the Shannon–Wiener curves, indicating that the database of 16S rRNA gene sequences was very abundant and reflected the vast majority of microbial information.

Microbial Population Structures in theIntestinal Lumen and Mucosa

The gut fecal samples showed significantly more diversity and richness than the mucosal samples (Table 2), and LM and MAM differed significantly (Fig.1A and B).

Significant differences between LM and MAM were identified in almost all populated phyla. Bacteroidetes (44.7%) andFirmicutes(42.2%) were the most strongly represented phyla in LM,followed by Proteobacteria(8.5%), whereas Proteobacteria (56.6%) was the most strongly represented phylum in MAM, followed by Firmicutes(20.2%) and Bacteroidetes(12.7%)($P<0.05$;Figure 2).

At the genus level, the relative abundances of *Escherichia–Shigella*, *Streptococcus*, *Clostridium* sensu stricto1,*Sphingomonas*, *Acinetobacter*, *Brevundimonas*, and *Enhydrobacter* were significantly greater in MAM than in LM,whereas those of *Bacteroides*, *Faecalibacterium*, *incertae sedis*, *Subdoligranulum*, *Pseudobutyrivibrio*, *Megasphaera*, *Parasutterella*, *Akkermansia*, *Alistipes*, and *Lachnospira* were significantly lower in MAM than in LM ($P < 0.05$).

Correlation with Age and BMI

Correlations with age and BMI were only detected in LM,and not in MAM. Fecal microbial diversity correlated significant positively with the age of the host (r=0.34, P=0.05). In LM, the proportions of phylum Firmicutes, class Clostridia(r = 0.398, P= 0.024), order Clostridiales (r = 0.398, P= 0.024), and family Ruminococcacea (r = 0.359, P= 0.043) correlated positively with age, whereas the proportion of family Bacteroides (r = –0.437, P= 0.012) correlated negatively with age.

In LM, class Bacteroidia(r= –0.367*, P*=0.039) and LM order Bacteroidales (r= –0.367,P=0.039) correlated negatively with BMI. In the fecal microbiota (LM),the proportions of phylum Firmicutes (r=0.480, P=0.018<0.05),class Coriobacteriia in phylum Actinobacteria(r=0.528, P=0.002), order Coriobacteria (r=0.504,P=0.007), family Coriobacteriaceae(r=0.504, P=0.007), genus *Collinsella* (r=0.435, P=0.013), and class Chloroplast inphylum Cyanobacteria(r=0.433, P=0.013) correlated positively with BMI.

Correlation with Mucosal Proteins BDNF, ZO1, TLR2, TLR4, AQP3, and AQP8

In a correlation analysis of bacterial abundance and the expression of mucosal proteins, distinct gut microbiota correlated with the expression ofBDNF (Table3), ZO1(Table4), TLR2(Table5), and TLR4(Table6). The bacteria that correlated with protein expression, age, and BMI mainly belonged to the phylum Firmicutes. The bacteria that correlated with BDNF expression, age, and BMI belonged to LM, whereas the bacteria that correlated with TLR4 and ZO1 expression belonged to MAM. Bacteria belonging to both LM and MAM correlated with TLR2 expression, and the trends in LM and MAM were consistent. Correlationswere detected with AQP8,but not with AQP3. In LM, the phylum Cyanobacteria, phylum Bacteroidetes, and genus *Prevotell*a correlated negatively with AQP8,and in MAM, the phylum Firmicutesand genus *Clostridiales* correlated negatively with AQP8. However, in MAM, the phylum Proteobacteriaand order Caulobacterales correlated positively with AQP8.

Discussion

Different habitats in the human body harbor distinct microbiota,which can be divided into different groups according to their anatomicallocations [9]. Mucosal samples were obtained from the large bowel before and after its preparation for an endoscopic procedure. Whether this bowel preparation affects the mucosal microbiota is still controversial. To avoid any interference, we collected the mucosal samples after bowel preparation. We used deep sequencing to determinethe bacterial compositions of the microbiota in the fecal samples and mucosal samples. More than 95% of the sequences in all the stool and mucosalsamples belonged to the three most popular bacterial phyla, Firmicutes, Bacteroidetes, and Proteobacteria. This isconsistent with the findings of previous studies, which showed that these phyla account for the majority of the gut microbiota in both stool and mucosal samples. The fecal samples displayed significantlygreaterbacterial diversity and richness than the mucosal samples,as in the study of Ringel et al. [2]. Comparing the proportions of the dominant bacterial taxa in the fecal and mucosal samples revealed significant differences. In this study,Proteobacteria was the predominant phylum in MAM.This differs from other reports, perhaps reflecting geographic differences, because it is well known that the Chinese diet and genetics are very differentfrom those in western countries, and these factors markedly influence the gut microbiota. Furthermore, MAM was sampled from a unique location, whereas LM was sampled from the whole intestinal microbiota, so MAM may have a more specific relationship with the host.

We performed a correlation analysis ofthe two bacterialpopulationsat the phylum, class, order, family, and genus levels,withsix proteins, host age, and host BMI. The results showed that although the Proteobacteria was the predominant phylum in MAM, the bacteria that correlated with specific proteins, age, and BMI mainly belonged to the phylum Firmicutes. In accordance with their distinct microbial compositions, LM and MAM showed different correlations. The bacteria that correlated with BDNF, age, and BMI belonged to LM.In contrast, the bacteria that correlated with TLR4 and ZO1 belonged to MAM. Bacteria that correlated with TLR2 belonged to both LM and MAM, and the trend was consistent in LM and MAM.

PeerJ Preprints | https://doi.org/10.7287/peerj.preprints.2526v1 | CC BY 4.0 Open Access | rec: 16 Oct 2016, publ: 16 Oct 2016

Age and BMI are important factors influencing the composition of the microbiota. Fecal microbial diversity correlated significantly positively with age,suggesting that microbial diversity increases with age. Members of the phylaBacteroidetes and Firmicutes were the main kinds of bacteria the microbiota, but they correlated oppositely with age and BMI. Based on the sequencing results, in LM,the proportions of the family Ruminococcaceae(belonging to classClostridia) andthe phylum Firmicutes correlated positively with age, whereasthe proportion of the family Bacteroides(belonging to phylum Bacteroidetes) correlated negatively with age. The phylum Bacteroides benefitshuman health, and these bacteria are reduced in older people[10]. It has been suggested that bacterial communities also undergo an aging process. In the LM microbiota, the phylum Firmicutes correlated positively with BMI,whereasthe phylum Bacteroidetes correlated negatively with BMI. Recent research has identified relationships between the bacterial composition of the gut microbiota and obesity. However, the results of studies of obesity and phylum-level changes in the gut microbiota are frequently contradictory,whichrequires explanation. These discrepancies may be attributable to methodological issues or geographic factors [11]. Interestingly, the phylumFirmicutes and class Coriobacteriia(belonging to the phylumActinobacteria) had opposite relationships with BMI and ZO1.As the numbers ofFirmicutes and Coriobacteriiaincreased, BMI increased, whereas the expression of ZO1 decreased. Many studies have shown that some bacteria are associated with BMI, and BMIsoutside the normal range are related to many diseases. Our results suggest that the interaction between bacteria and BMI maybe related to the expression of ZO1.

Like age and BMI, the bacteria that correlated with BDNF expression belonged to LM, but these bacteria only belonged to the phylum Firmicutes. Interestingly, all the bacteria that correlated with BDNF expression also correlated with TLR2 expression, and with the same trends. As the bacteria in the genus *Faecallibacterium*(family Ruminococcaceae, order Clostridiales, class Clostridia), the genus *Lachnospira* (familyLachnospiraceae,order Clostridiales, class Clostridia), and the order Lactobacillales(class Bacilli)increased, the expression of BDNF and TLR2 decreased. BDNF also correlated positively with TLR2. Although positive correlations also existed between BDNF and TLR4 and between TLR2 and TLR4, the bacteria associated with TLR4 expression differed greatly from those related tothe expression of BDNF and TLR2. The bacteria that correlated with TLR4 were only found in MAM. Some bacteria that negatively correlated with TLR2 were also found in MAM, but they only belonged to the class Bacilli, whereas TLR4 expression was mainly associated with the class Clostridia. The enteric commensal bacteria of the genus*Faecallibacterium*,which belongs to the Clostridium group,exert an anti-inflammatory effect.In the present study, *Faecallibacterium* correlated negatively with the expression of BDNF, TLR2, and TLR4. Similarly, the order Lactobacillales(class Bacilli)also correlated negatively with BDNF, TLR2, and TLR4. Lactobacillales and *Faecallibacterium* may display similar effects because Lactobacillales can cause the numbers of*Faecallibacterium*to increase and the TLRs may exert an anti-inflammatory effect.

Although the phylumProteobacteriawas the most strongly represented bacterial taxon in MAM, only a few bacteria from the Proteobacteria correlated with the expression of specific proteins: MAM members of the class Betaproteobacteria correlated positively with ZO1 and MAM members of the family Mitochndric(order Rickettsiales, class Alphaproteobacteria) correlated positively with TLR2. The genus*Haemophilus*(family Pasteurellaceae, order Pasteurellales, class Gammaproteobacteria)contains common neutral or pathogenic bacteria, andin MAM, genus*Haemophilus* correlated negatively with BDNF, TLR2, and TLR4, and in LM,*Haemophilus* also correlated negatively with TLR2. Round et al. showed that unlike pathogens whose TLR ligands trigger inflammation, some commensal bacteria exploit the TLR pathway to actively suppress immune reactions [12]. Our findings indicate that commensal microbes avoid activating TLR and that the pathogenicity of *Haemophilus*may be related to the inhibition of the primary immune response.

ZO1 is an important tight junction protein.Six MAM bacteria correlated positively with ZO1 expression. Therefore, increases in these bacteria may increase the levels of ZO1, partly restoring the function of the intestinal barrier. AQPs areessential proteins in water metabolism, and the different AQP proteins are expressed in different locations, with AQP3 and AQP8 present in the colon. In this study,bacteria correlated with AQP8,but not with AQP3. Fecal Cyanobacteria (phylum) correlated negatively with AQP8. In LM, the genus *Prevotella* genus (phylum Bacteroidetes) correlated negatively with AQP8, as did the genus*Clostridium*(phylum Firmicutes) in MAM, whereasin MAM,members of the order Caulobacterales(phylum Proteobacteria) correlated positively with AQP8. These results suggest that AQP8 participates in water absorptionby the microbiota.

The microbiota show geographic differences.Although our data for Chinese individualsshowed ahigher abundance ofProteobacteria, members of the phylum Firmicutes correlatedmoststronglywith the parameters and proteins tested. Bacteria can be classed by phylum, class, order, genus, or species, and recent research has predominantlyfocused on the phylum and genus levels. However, in the present study, many genera belonging to the same order displayed the same trends. In examining the functions of bacteria, we must consider the taxonomic level, and the order level may be the best level at which to study bacterial function. The components of MAM and LM were very different, and few correlations were shared by both populations of bacteria. Interestingly, correlations with systemic indicessuch as age, BMI, and BDNF expressionwere mainly observed among the LM bacteria, whereas correlations with systemic and local indices, such as TLR2 expression, were observed in both MAM and LM. The protein ZO1,which usually exerts a local effect, mainly correlated with bacteria in MAM. These results suggest that the functions of bacteria are closely related to their sites of occurrence in the gut. In conclusion, the microbiota of LM and MAM differ. Because microbial population structure reflects function, the different bacteria colonizing different locations in the gastrointestinal tract play different roles by regulating the expression of different proteins.

References:

1 **Kataoka K**. The intestinal microbiota and its role in human health and disease.

J Med Invest. 2016;63(1–2):27–37. [PMID: 27040049 DOI: 10.2152/jmi.63.27.]

2 **Ringel Y**, Maharshak N, Ringel-Kulka T, Wolber EA, Sartor RB, Carroll IM. High throughput sequencing reveals distinct microbial populations within the mucosal and luminal niches in healthy individuals. Gut Microbes. 2015;6(3):173-181.[PMID: 25915459 DOI:10.1080/19490976]

3**Sundin J,** Rangel I, Fuentes S, Heikamp-de Jong I, Hultgren-Hörnquist E, de VosWM, Brummer RJ. Altered faecal and mucosal microbial composition in post-infectious irritable bowel syndrome patients correlates with mucosallymphocyte phenotypes and psychological distress. Aliment Pharmacol Ther. 2015;41(4):342-351. [PMID:25521822 DOI: 10.1111/apt.13055.]

4 **Piche T.** Tight junctions and IBS--the link between epithelial permeability,low-grade inflammation, and symptom generation? Neurogastroenterol Motil. 2014;26(3):296-302.[PMID: 24548256.DOI: 10.1111/nmo.12315]

5 **Wang P**, Chen F, Du C, Li C, Yu Y, Zuo X, Li Y. Increased production of BDNF in colonic epithelial cells induced by fecal supernatants from diarrheic IBS patients. Sci Rep. 2015;5:10121.[PMID:25998025 DOI: 10.1038/srep10121]

6 **de Kivit S**, Tobin MC, Forsyth CB, Keshavarzian A, Landay AL. Regulation of Intestinal Immune Responses through TLR Activation: Implications for Pro- and Prebiotics. Front Immunol. 2014;5:60.[PMID: 24600450 DOI: 10.3389/fimmu]

7 **Guttman JA**, Samji FN, Li Y, Deng W, Lin A, Finlay BB. Aquaporins contribute to diarrhoea caused by attaching and effacing bacterial pathogens. Cell Microbiol. 2007;9(1):131-141.[PMID: 16889624]

8 **Metzler-Zebeli BU,** Mann E, Schmitz-Esser S, Wagner M, Ritzmann M, Zebeli Q. Changing dietary Calcium-Phosphorus level and cereal source selectively alters abundance of bacteria and metabolites in the upper gastrointestinal tracts of weaned pigs. Appl EnvironMicrobiol. 2013;79(23):7264-7272.[PMID:24038702 DOI: 10.1128/AEM.02691-13]

9 **Ling Z,** Liu X, Luo Y, Yuan L, Nelson KE, Wang Y, Xiang C, Li L. Pyrosequencinganalysis of the human microbiota of healthy Chinese undergraduates. BMC Genomics.2013;10;14:390.[PMID: 23758874 DOI: 10.1186/1471-2164-14-390]

10 **Wang F**, Yu T, Huang G, Cai D, Liang X, Su H, Zhu Z, Li D, Yang Y, Shen P, Mao R, Yu L, Zhao M, Li Q. Gut microbiota community and its assembly associated with age and diet in chinese centenarians. J Microbiol Biotechnol. 2015;25(8):1195-1204. [PMID: 25839332 DOI: 10.4014/jmb.1410.10014]

11 **Escobar JS**, Klotz B, Valdes BE, Agudelo GM: The gut microbiota of Colombians differs from that of Americans, Europeans and Asians. BMC Microbiol. 2014;14:311.[PMID: 25495462

DOI: 10.1186/s12866-014-0311-6]

12 **Round JL,** Lee SM, Li J, Tran G, Jabri B, Chatila TA, Mazmanian SK. The Toll-Like receptor 2 pathway establishes colonization by a commensal of the human microbiota. Science. 2011;332(6032):974-977.[PMID: 21512004 DOI: 10.1126/science.1206095]

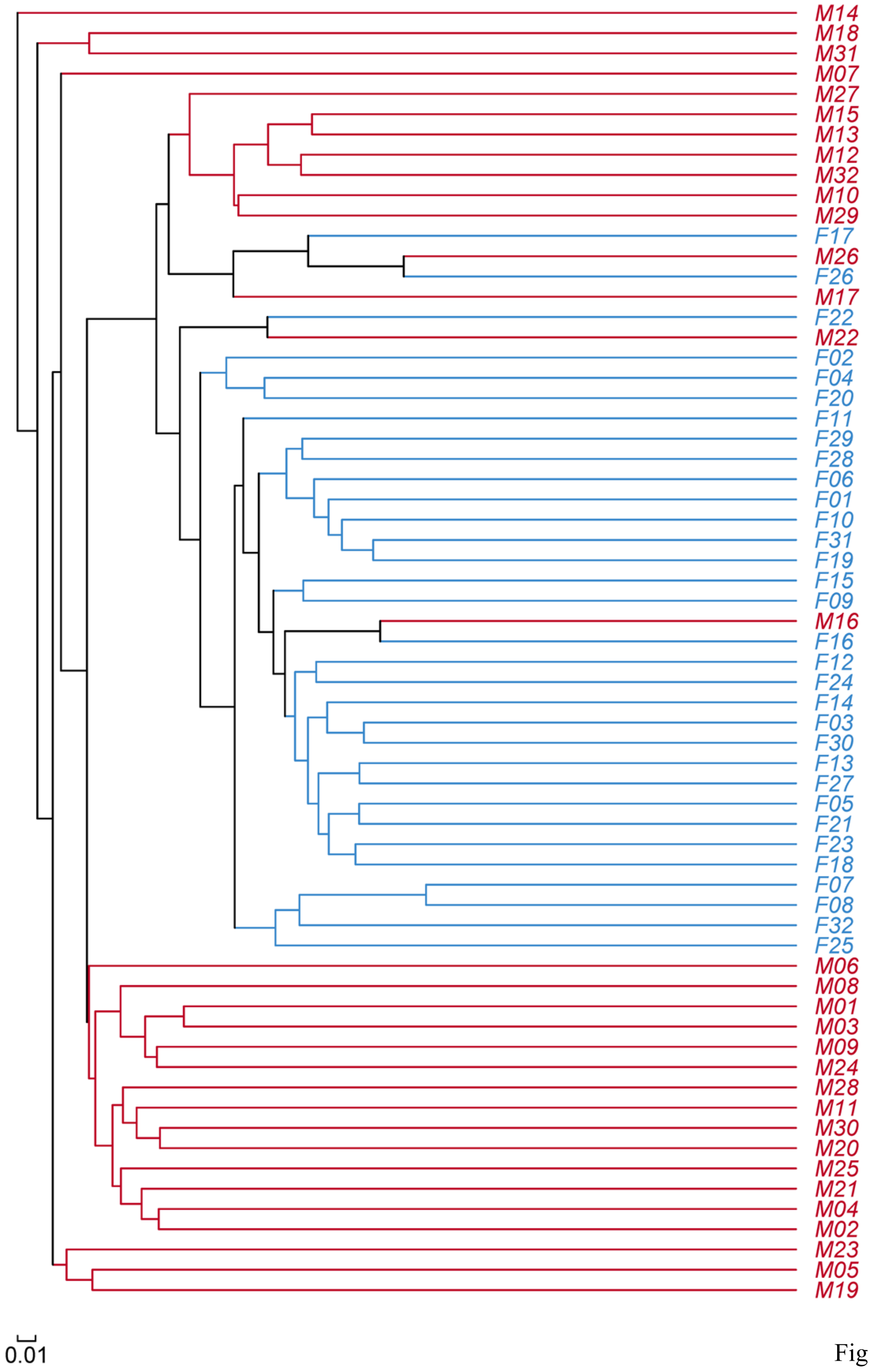

Fig1A

374

375

PeerJ Preprints | https://doi.org/10.7287/peerj.preprints.2526v1 | CC BY 4.0 Open Access | rec: 16 Oct 2016, publ: 16 Oct 2016

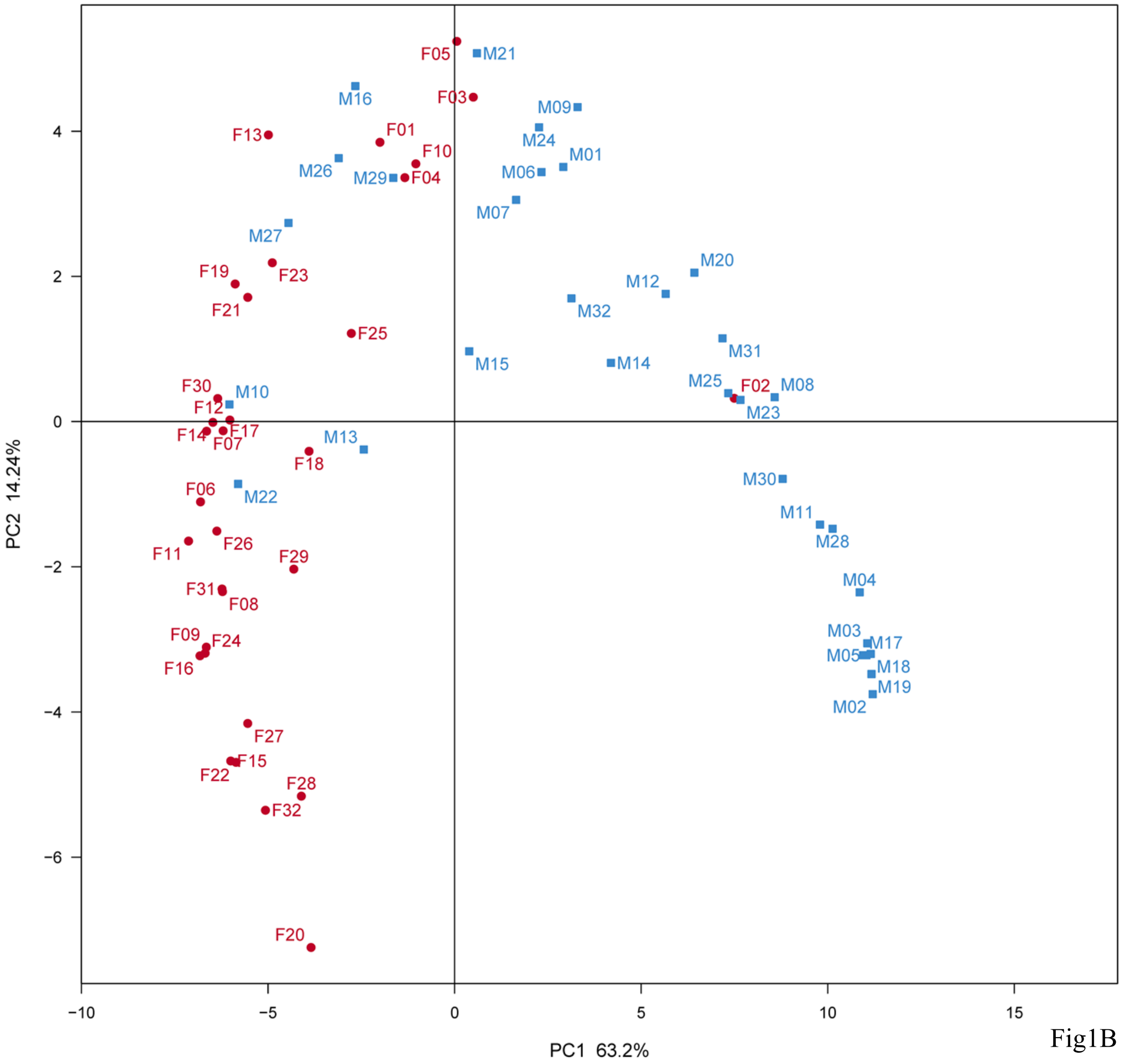

Fig1. 16S rRNA gene surveys reveal a clear separation of the LM and MAM.

(A) Dendrogram obtained with complete linkage hierarchical clustering of the samples from stool and mucosa based on the total OTUs. (B) Principal coordinate analysis (PCoA） plot based on the weighted UniFrac metric.

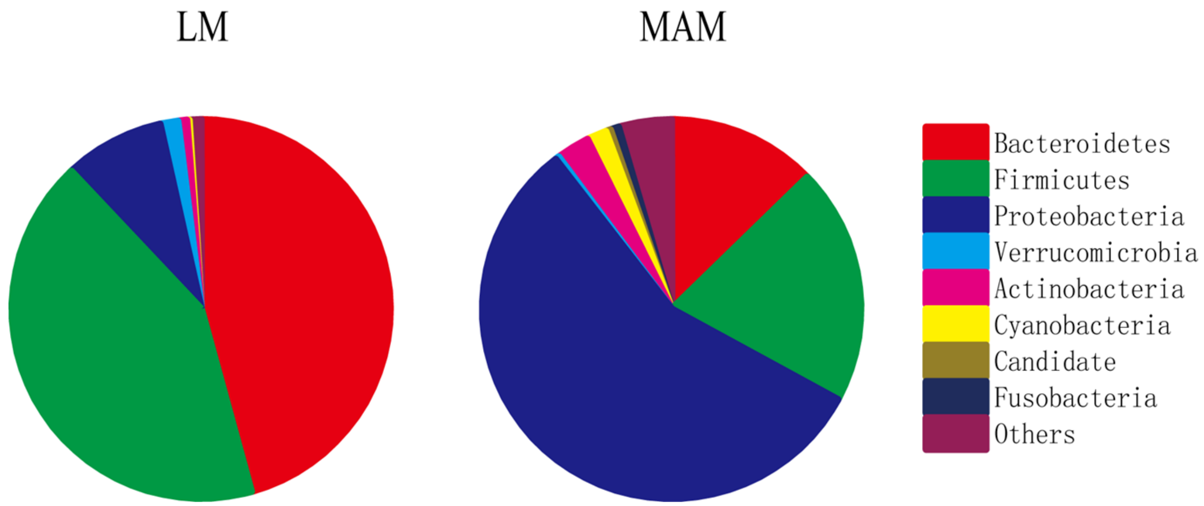

Fig2. Relative abundances of bacterial phylum in the stool and mucosa samples. Statistically significant differences in all phylum($p < 0.05$).

Table1 Quantitative polymerase chain reaction (qPCR) primer used in this study to enumerate specific gene

		Primer Sequence 5’-3’
BDNF	F	AGGTGGCTCTGGAATGACAT
	R	GGCATAAGTCGGCTTGAGTG
ZO-1	F	CAGTGCCTAAAGCTATTCCTGTGA
	R	CTATGGAACTCAGCACGCCC
TLR2	F	TGATGCTGCCATTCTCATTC
	R	CGCAGCTCTCAGATTTACCC
TLR4	F	CAGGGCTTTTCTGAGTCGTC
	R	TGAGCAGTCGTGCTGGTATC
AQP3	F	AGACAGCCCCTTCAGGATTT
	R	TCCCTTGCCCTGAATATCTG
AQP8	F	GGAATATCAGTGGTGGACACT
	R	CCAATGAAGCACCTAATGAGC

Table2 Comparison of the riches and diversity of MAM and LM

	ace	chao	shannon	simpson
MAM	160.72±51.62	151.56±48.63	2.29±1.24	0.33±0.32

LM	195.50±42.04	188.65±42.70	2.901±0.54	0.13±0.07
P	0.004**	0.002**	0.012*	0.001**

**$P<0.01$ *$P<0.05$

Table3 Bacterial taxa that significantly correlated with BDNF

	phylum	class	order	familly	genus
LM	Firmicutes	Clostridia*	Clostridiales*	Ruminococcacea	Faecallibacterium*
		r=-0.415, p=0.018	r=-0.415,P=0.018		R=-0.451,P=0.01
LM	Firmicutes	Clostridia*	Clostridiales*	Lachnospiracea*	Lachnospira**
		r=-0.415, p=0.018	r=-0.415,P=0.018	R=-0.434,P=0.013	R=-0.588,P=0.00
LM	Firmicutes	Bacilli**	Lactobacillal**	Lactobacillace**	Lactobacillus**
		r=-0.523, p=0.002	r=-0.524,p=0.002	R=-0.655,P=0.004	R=-0.546,P=0.001

Table4 Bacterial taxa that significantly correlated with ZO1

	phylum	class	order	familly	genus
MAM	Firmicutes	Clostridia*	Clostridiales*	Ruminococcaceae*	Subdohgranulum*
		R=0.384,P=0.03	R=0.386,P=0.02	R=0.297,P=0.099	R=0.444,P=0.01
MAM	Firmicutes	Clostridia*	Clostridiales*	Lachnospiraceae*	Dorea**
		R=0.384,P=0.03	R=0.386,P=0.02	R=0.264,P=0.144	R=0.513,P=0.00
MAM	Firmicutes	Negativicutes*	Selenomonadales*	Veillorellaceae	Megamonas**
		R=0.384,P=0.044	R=0.384,P=0.04		R=0.456 P=0.00
MAM	Bacteroidetes	Bacteroidia	Bacteroidia	Bacteroidoceae	Bacteroides
		R=0.423,P=0.016	R=0.423,P=0.016	R=0.407,P=0.02	P=0.407,R=0.02
MAM	Bacteroidetes	Flavobacteriia	Flavobacteriia	Porphyromonadaceae	Parabacteroides
		R=0.418,P=0.03	R=0.418,P=0.03	R=0.189,P=0.337	R=0.443,P=0.02
MAM	Proteobacteria	Betaproteobacteria**	Burkholderiales**	Alcaligenaceae**	-

PeerJ Preprints | https://doi.org/10.7287/peerj.preprints.2526v1 | CC BY 4.0 Open Access | rec: 16 Oct 2016, publ: 16 Oct 2016

		R=0.472,P=0.00	R=0.532,P=0.00	R=0.594,P=0.00	
MAM	Proteobacteria	Betaproteobacteria**	Burkholderiales**	Oxalobacteraceae*	-
		R=0.472,P=0.00	R=0.532,P=0.00	R=0.553,P=0.05	
LM	Firmicutes*	-	-	-	-
	R=-0.427 p=0.037				
LM	Atinobacteria	Coriobacteria*	Coriobacteria*	Coriobacteriac*	Collinsella*
		R=-0.393,P=0.026	R=-0.421,P=0.029	R=-0.421,P=0.029	R=-0.387,R=0.029

Table5 Bacterial taxa that significantly correlated with TLR2

	phylum	class	order	familly	genus
LM	Firmicutes	Clostridia	Clostridiales	Ruminococcaceae	Faecalibacterium
		R=-0.522,P=0.00	R=-0.522,P=0.00	R=-0.486,P=0.00	R=-0.593,P=0.00
LM	Firmicutes	Clostridia	Clostridiales	Lachnospiraces	Lachnospira
		R=-0.522,P=0.00	R=-0.522,P=0.00	R=-0.392,P=0.02	R=-0.400,P=0.02
LM	Firmicutes	Bacilli	Lactobacillal	Streptococcaceae	Streptococcus
		R=-0.390,P=0.02	R=-0.388,P=0.02	R=-0.466,P=0.00	R=-0.479,P=0.00
MAM	Firmicutes	Bacilli	Lactobacillal	Streptococcaceae	Streptococcus
		R=-0.390,P=0.02	R=-0.367,P=0.04	R=-0.533,P=0.00	R=-0.513,P=0.00
LM	Firmicutes	Bacilli	Lactobacillal	Carnobacteriaceae	GranulicatellA
		R=-0.390,P=0.02	R=-0.388,P=0.02		R=-0.373,P=0.03
MAM	Firmicutes	Bacilli	Lactobacillal	Carnobacteriaceae	GranulicatellA

PeerJ Preprints | https://doi.org/10.7287/peerj.preprints.2526v1 | CC BY 4.0 Open Access | rec: 16 Oct 2016, publ: 16 Oct 2016

		R=-0.390,P=0.02	R=-0.388,P=0.02	R=-0.696,P=0.00	R=-0.541,P=0.00
LM	Proteobacteria	Gammaproteobacteria	Pasteurellales R=-0.560,P=0.00	Pasteurellsceae R=-0.560,P=0.00	Haemophilum R=-0.457,P=0.00
MAM	Proteobacteria	Gammaproteobacteria	Pasteurellales R=-0.650,P=0.00	Pasteurellsceae R=-0.565,P=0.00	Haemophilum R=-0.509,P=0.00
MAM	Proteobacteria	alphaProteobacteria	Rickettsioles	Mitochndric R=0.605,P=0.03	-
MAM	chloroflexi	Ktedonobacteria R=-0.696,P=0.01	Ktedonobactera R=-0.727,P=0.04	-	-
MAM	SAR	Foraminifere R=0.522,P=0.00	-	-	-

399 Table6 Bacterial taxa that significantly correlated with TLR4

	phylum	class	order	familly	genus
MAM	Firmicutes	Clostridia*	Clostridiales*	Ruminococcaceae**	Ruminococcus*
		R=-0.371,P=0.03	R=-0.369,P=0.03	R=-0.515,P=0.00	R=-0.442,P=0.03
MAM	Firmicutes	Clostridia*	Clostridiales*	Ruminococcaceae**	Faecalibacterium**
		R=-0.371,P=0.03	R=-0.369,P=0.03	R=-0.515,P=0.00	R=-0.481,P=0.00
MAM	Firmicutes	Clostridia*	Clostridiales*	Lachnospiraces	Incertae Sedis*
		R=-0.371,P=0.03	R=-0.369,P=0.03		R=-0.370,P=0.03
MAM	Firmicutes	Clostridia*	Clostridiales*	Lachnospiraces	Blautia*
		R=-0.371,P=0.03	R=-0.369,P=0.03		R=-0.355,P=0.046
MAM	Firmicutes	Clostridia*	Clostridiales*	Peptostreptoco*	-
		R=-0.371,P=0.03	R=-0.369,P=0.03	R=-0.445,P=0.01	
MAM	Firmicutes	Bacilli	Lactobacillal	Streptococcacea*	Streptococcus*

PeerJ Preprints | https://doi.org/10.7287/peerj.preprints.2526v1 | CC BY 4.0 Open Access | rec: 16 Oct 2016, publ: 16 Oct 2016

				R=-0.383,P=0.04	R=-0.385,P=0.03
MAM	Verrucomicrobia	Verricomicrobiae* R=-0.819,P=0.046	Verrucomicrobiales	Verrucomicrobiales* r=-0.815,p=0.025	-
MAM	Proteobacteria	Gammaproteobacteria	Pasteurellales* R=-0.446,P=0.03	Pasteurellsceae** R=-0.457,P=0.00	Haemophilum* R=-0.446,P=0.01
MAM	Atinobacteria	Atinobacteria	Bifidobacteria* R=-0.485,P=0.01	Bifidobacteriaceae* R=-0.416,P=0.03	Bifidobacteriaceae* R=-0.403,P=0.02