Microbiome insights into the perforated duodenal ulcer via metagenomic sequencing

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Abstract

Background Perforated duodenal ulcer is a common condition. The associated microbiome and relationship with treatment success remain to be explored in addition to the well recognized role of Helicobacter pylori.

Methods Metagenomic sequencing were used to profile the microbes underlying duodenal ulcer. The metagenome results of 6 duodenal ulcer samples (3 ulcer foci and 3 sites around the ulcer foci) were obtained and compared.

Findings Ulcer patients and healthy control have different gut-type. Streptococcus mitis was highly enriched in duodenal ulcer patients.

Interpretation The dysbiosis of microbiomes might underlie the duodenal ulcer, while the increased abundance of Streptococcus mitis is a novel finding. Whether there is causal link between S.mitis and duodenal ulcer remains to be determined.

Significance Of This Study

What is already known on this subject?

As part of the digestive duct, duodenum populates abundant commensal microbes. *H. pylori* was the widely and only recognized causative agent of duodenal ulcer.

What are the new findings?

This represents the first report on the gut microbiome of duodenum or duodenal ulcer. *Streptococcus mitis* is novel finding in the duodenal ulcer. Ulcer patients and healthy control have different gut type. The gut microbiota dysbiosis might play an unexpected role in the disease.

How might it impact on clinical practice in the foreseeable future?

The *Streptococcus mitis* and dysbiosis of microbiota can be new direction for the duodenal ulcer study.

Introduction

Intestinal tract is one of the most important digestive organ closely relevant to human health [1, 2]. The human digestive tract is populated by a large number of microbes [2], with cell numbers (10^{14}) exceed that of body cells (10^{13}) by a factor of ten [3]. These “aboriginal inhabitants” might protect the host from damage. Activation of Toll-like receptors (TLRs) by commensal microflora is critical for the protection against gut injury and associated mortality, for these bacteria are recognized by TLRs under physiological

status [4]. Gut microbiota affects multiple aspects of host, such as energy harvest from the diet and energy storage [5], intestinal angiogenesis [6] and host immunity [7].

The gut microbes can contribute to resist numerous invading pathogens. *Bacillus bifidus* can protect or resist the ulcerative inflammation in gut [8], while the *Escherichia coli* often causes diarrhea [9]. The gut microbes are closed implicated in human health [10–12]. The immunologic response and metabolism of endogenous or exogenous compounds in the liver are interweaved with the gut microbes[12]. *E. coli* evolution abilityis associated with the gut microbiota homeostasis [13]. Inflammatory bowel diseases, Crohn's disease and ulcerative colitis, are chronic idiopathic disorders causing inflammation of the gastro-intestinal tract [14]. It's reported that more than 60 percent population was carrier of *Helicobacter pylori,* the culprit of superficial gastritis. *H. pylori* is an opportunistic pathogen[15, 16]. The duodenum, one of the most important digestive organs, is located between stomach and jejunum. *H. pylori* was widely considered as the culprit of these gastroduodenal disorders [17, 18]. Few direct evidences can be found for its role in duodenal ulcer. Duodenal ulcer, one of the common digestive diseases, was considered as the result of convulsion in stomach and duodenum. The microbes implicated in duodenal ulcer are poorly studies.

In this study, metagenome sequencing was performed for 3 duodenal ulcer patients, six samples were collected including 3 ulcer sites and 3 sites distant from the ulcer. Each patient contributed two samples, one from the ulcerate site, the other distant from the ulcer foci as control. Metagenomic data showed unexpected enrichment of *Streptococcus mitis* in the ulcer sites.

Materials And Methods

DNA Extraction and detection

Samples separated from three patients were harvested and sent to Shanghai Biotechnology Corporation for metagenomic sequencing. DNA was extracted according to conventional protocol.

DNA degradation and potential contamination were monitored on 1% agarose gels. DNA purity (OD_{260}/OD_{280}, OD_{260}/OD_{230}) was monitored by using the NanoPhotometer® spectrophotometer (IMPLEN, CA, USA). DNA concentration was measured using Qubit® dsDNA Assay Kit in Qubit® 2.0 Flurometer (Life Technologies, CA, USA). OD value is between 1.8~2.0, DNA contents above 1μg are used to construct library.

Library construction

A total amount of 1μg DNA per sample was used as input material for the DNA sample preparations. Sequencing libraries were generated using NEBNext® Ultra™ DNA Library Prep Kit for Illumina (NEB, USA) following manufacturer's recommendations and index codes were added to attribute sequences to each sample. Briefly, the DNA sample was fragmented by sonication to a size of 350bp, then DNA fragments

were end-polished, A-tailed, and ligated with the full-length adaptor for Illumina sequencing with further PCR amplification. At last, PCR products were purified (AMPure XP system) and libraries were analyzed for size distribution by Bioanalyzer and quantified using real-time PCR.

Data Analysis

The clustering of the index-coded samples was performed on a cBot Cluster Generation System according to the manufacturer's instructions. After cluster generation, the library preparations were sequenced on an Illumina HiSeq platform and paired-end reads were generated. The Clean Data is assembled and analyst[19] by SOAPdenovo software (V2.04, http://soap.genomics.org.cn/soapdenovo.html), the parameters[20, 21] are as follows: -d 1, -M 3, -R, -u, -F, -K 55; To the samples taken from complex environment, such as water, soil and so on, MEGAHIT software (v1.0.4-beta) could be used to assemble the Clean Data and the parameters[22] are -presets meta-large (—min-count 2—k-min 27—k-max 87—k-step 10); then interrupted the assembled Scaftigs from N connection and leave the Scaftigs without N[20]. All samples' Clean Data are compared to each Scaffolds respectively by SoapAligner software (soap 2.21) to acquire the PE reads not used and the parameters[20] are: identity ≥ 90%, -m 200 -x 400.

Gene prediction and abundance analysis

The Scaftigs (≥ 500 bp) assembled from both single and mixed are all predicted for the ORF by MetaGeneMark (V2.10, http://topaz.gatech.edu/GeneMark/) software, and filtered the length information shorter than 100nt from the predicted result with default parameters. For ORF predicted, CD-HIT software (V4.5.8, http://www.bioinformatics.org/cd-hit) is adopted to redundancy and obtain the unique initial gene catalogue (the genes here refers to the nucleotide sequences coded by unique and continuous genes[23]), the parameters option[23] are -c 0.95, -G 0, -aS 0.9, -g 1, -d 0. The Clean Data of each sample is mapped to initial gene catalogue using SoapAligner (soap 2.21) and get the number of reads to which genes mapped in each sample with following parameters: -m 200, -x 400, identity ≥ 95%. Filter the gene with reads ≤ 2 in each sample and obtain the gene catalogue (Unigenes) for subsequent analysis. The abundance of each gene per sample was based on the number of mapped reads and the length of gene. The format is, r represents the number of reads mapped to the genes and L represents the gene length. The basic information statistic, core-pan gene analysis, correlation analysis of samples and van Fig analysis of number of genes are all based on the abundance of each gene per sample in gene catalogue.

Taxonomy prediction

DIAMOND software (V0.7.9, https://github.com/bbuchfink/diamond/) is used to BLAST the Unigenes to the sequences of Bacteria, Fungi, Archaea and Viruses which are all extracted from the NR database (Version: 20161115, https://www.ncbi.nlm.nih.gov/) of NCBI with the parameters: blastp, -e 1e−5. For the aligned results of each sequence, as each sequence may have multiple aligned results, the result with an

e value ≤ the smallest e value * 10 by the LCA algorithm for the system classification of MEGAN software was used to determine the species annotation of sequences. The table containing the genes numbers and abundance per sample in each taxonomy hierarchy (kingdom, phylum, class, order, family, genus, species) is based on the LCA annotation result and the gene abundance table. The abundance of a specie in one sample equal the sum of the gene abundance annotated for the species; the gene number of a species in a sample equates to the number of genes with nonzero abundance. Kmoa analysis, the representation of relative abundance, the exhibition of abundance cluster heat map, PCA (R ade4 package, Version 2.15.3) and NMDS (R vegan package, Version 2.15.3) decrease-dimension analysis are based on the abundance table of each taxonomic hierarchy. The difference between groups is tested by Anosim analysis (R vegan package, Version 2.15.3). Metastats and LEfSe analysis are used to look for the different species between groups. Permutation test between groups is used in Metastats analysis for each taxonomy and get the *P* value, then use Benjamini and Hochberg False Discovery Rate to correct P value and acquire q value. LEfSe analysis is conducted by LEfSe software (the default LDA score is 3)

Common functional database annotations

DIAMOND software (V0.7.9) was used to BLAST Unigenes to functional database with the parameters: blastp, -e 1e−5. Functional database including KEGG database (Version 201609, http://www.kegg.jp/kegg/), eggNOG database (Version 4.5, http://eggnogdb.embl.de/#/app/home), CAZy database (Version 20150704, http://www.cazy.org/). For the BLAST result of each sequence, the best Blast Hit is used for subsequent analysis. Statistic of the relative abundance of different functional hierarchy, the relative abundance of each functional hierarchy equal to the sum of relative abundance annotated to that functional level. Based on the function annotation result and gene abundance table, the gene number table of each sample in each taxonomy hierarchy is obtained. The gene number of a function in a sample equal the gene number that annotated to this function and the abundance is nonzero. Based on the abundance table of each taxonomy hierarchy, the count of annotated gene numbers, general relative abundance, the abundance cluster heat map and the decrease-dimension analysis of PCA and NMDS are conducted, as well as the Anosim analysis of the difference between groups (inside) based on functional abundance. The metabolic pathways, Metatat and LEfSe analysis between groups are performed.

Resistance gene annotation

DIAMOND software (V0.7.9) was used to align the Unigenes to ARDB database (http://ardb.cbcb.umd.edu/) with the parameters: blastp, -e 1e−5. Filter the aligned result and choose the identity value bigger than the lowest identity value from the aligned result of each sequence, to make sure the resistance gene annotation is reliable. Based on the aligned result, count the relative abundance of each resistance gene. The bar charts and cluster heat map were used to visualize the abundance of resistance genes and associated species.

Results

The duodenal ulcer microbiota was significantly altered by metagenomic study.

To explore the metagenomic change of duodenal ulcer patients, 6 samples (3 ulcer samples and 3 controls) were subjected to metagenomic sequencing. Consistent with the role of gut microbiota change in metabolic disorders [10], multiple central metabolic pathways were underrepresented in duodenal ulcer patients, such as ABC transporters, amino acids biosynthesis, carbon metabolism and pyrimidine metabolism (Figure 1A). These underrepresented pathways were highlighted (Figure 1B). D-glutamine and D-glutamate metabolism, sphingolipid metabolism and nitrotoluene degradation were significantly decreased. Tetracycline biosynthesis pathway was enriched 3000 folds. Bacteria population density was auto-regulated [24]. The enrichment of tetracycline biosynthesis pathway and antibiotics resistance genes (with abundance over 0.03%) indicated the specifically proliferating of microbes bearing these genes (Figure 1C). The percentage of duodenum microbes is similar between ulcer site and site near ulcer (Figure 2A), except the virus. Bacteria are predominant (77%–92%) (Figure 2A). Among the top10 species which contribute 51%, *Gemella haemolysans* (10.6%), *Gemella sanguinis* (7.8%), *Fusobacterium periodonticum* (5.9%), *Streptococcus mitis* (7.3%), *Prevotella sp. Oral taxon 473* (7.5%) are very prominent. *Veillonella* unclassified (6.6%), *Neisseria* unclassified (3.6%), *Neisseria flavescens* (3.4%), *Porphyromonas sp. Oral texon 279* (3.7%) are predominant in the health control (Figure 2B). There is no overlap for the top 10 species between patients and health control. This comparison indicated significant change of microbiota during duodenal ulcer.

Streptococcus mitis was unexpectedly enriched in duodenal ulcer site samples

For the altered bacteria species, we corrected data about several pathogens in 353 patients. The directed enrich of *S. mitis* in patients was observed (Figure 3 and Table 1). The enhanced survival of *S. mitis* in old patients might hinted link between the duodenal ulcer and *S. mitis*

Discussion

Gut microbiota is increasingly recognized as key player in host healthy [12, 20], largely due to close physical contact [25] and modulating the differentiation of immune cell subsets [26], the production of cytokines and chemokines to influence the T cell repertoire of the intestine and surrounding tissue, and the production of soluble immune mediator IgA [27, 28]. *Bifidobacterium* abundance was changed by gene-diet interaction [29]. However, to our knowledge, there is no reports on the gut microbiota of duodenum or duodenal ulcer.

In this study, metagenomic results showed that bacterium instead of virus or fungi dominated the duodenum (Figure 2A). Though both health control and ulcer sites are populated with bacteria, significant difference exists. *S. salivarius, S. parasanguinis, Megasphaera micronuciformis, Porphyromonas endodontails* are abundant in health control (Figure 2B), *G. sanguinis, Fusobacterium periodonticum, G.*

haemolysans, S. mitis [30], bacteria frequently found in oral cavity, were found also dominant in ulcer sites (Figure 2B). In general, the top 10 species of the ulcer sites (US) are more abundant than TOP 10 in health control (NUS) (Figure 2B). The duodenal ulcer was accompanied by abundance change of dominant bacteria. Some hemolytic bacteria were found in duodenal ulcer, while absent in health control. *G. haemolysans, G. sanguinis, S. mitis* might underlie the duodenal ulcer. Gut type is a recently well recognized conception in gut microbiota field. *Prevotella, Bacteroides* and *Ruminococcaceae* were core for gut types identification [31]. In our results, *Prevotella* are predominant both in patients (6.9%) and health (6.9%), *Bacteroides* is presentin patients (0.2%) and absent in health. *Ruminococcaceae* (0. 0008%) was found in patients only. The result suggested that ulcer patients and healthy control have different gut type.

S. mitis is one of mitis group *Streptococci,* which are abundant members of the microbiota on all surfaces in the oral cavity and pharynx birth and through lifespan [30]. As an oral parasite, *Streptococcus mitis* is opportunistic pathogens implicated in dental caries [32], subacute bacterial endocarditis [33], brain abscesses [34]. The role of *S. mitis* in duodenal ulcer warrants further study.

The copies of some bacterial genes involved in metabolic pathways are low in ulcer patients. The underrepresented KEGG [35] pathways are ABC transporters [36], amino acids biosynthesis [37] and carbon metabolism [38] (Figure 2A). The underlying mechanism of action remains elusive. The dysregulated metabolism of microbiota might underlie the duodenal ulcer.

The change of bacteria location was previously reported to be involved in diseases, such as cephalomeningitis, cirrhosis. Microbial metabolite imidazole propionate might contribute to the pathogenesis of type 2 diabetes by impairing insulin signal [39]. The presence of several conventional oral bacteria such as *S. mitis* in duodenal ulcer might be novel pathogen of duodenum ulcer, or underlying the dysbiosis of microbiota, resulted in significant abundance change (Figure 2B), culminated in ulcer. More studies into the role and mechanism of action of *S. mitis* might offer new insights into the duodenal ulcer.

Declarations

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Declaration of Interests

None to declare.

Author Contributions

X. H., X. D., J.X, S. Y., and J. L. performed the experiments. X. H., and X. D., J. N. analyzed the data. S.M diagnosed the patients, collected samples, for all clinical related ethic approval and part of data analysis. X. H. and X. D., J. X. designed the study and wrote the paper. All authors have read and approved the manuscript.

Ethics Statement

Research conducted for this study was performed in accordance with approvals from the Institutional Review Board at the Hospital. All participants provided written informed consent prior to participation in the study.

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Table

Table 1. The age difference for the distribution of microbes among 353 patients

Species	Counts	Ages			
		0-20	20-39	40-60	>60
Streptococcus mitis	6	0	0	1	5
Neisseria	1	1	0	0	0
Pseudomonas aeruginosa	2	0	0	0	2
Klebsiella pneumoniae	10	0	1	0	9
Escherichia coli	12	0	2	5	5
Acinetobacter baumannii	1	0	0	1	0
Enterobacter cloacae	9	0	3	1	5

Figures

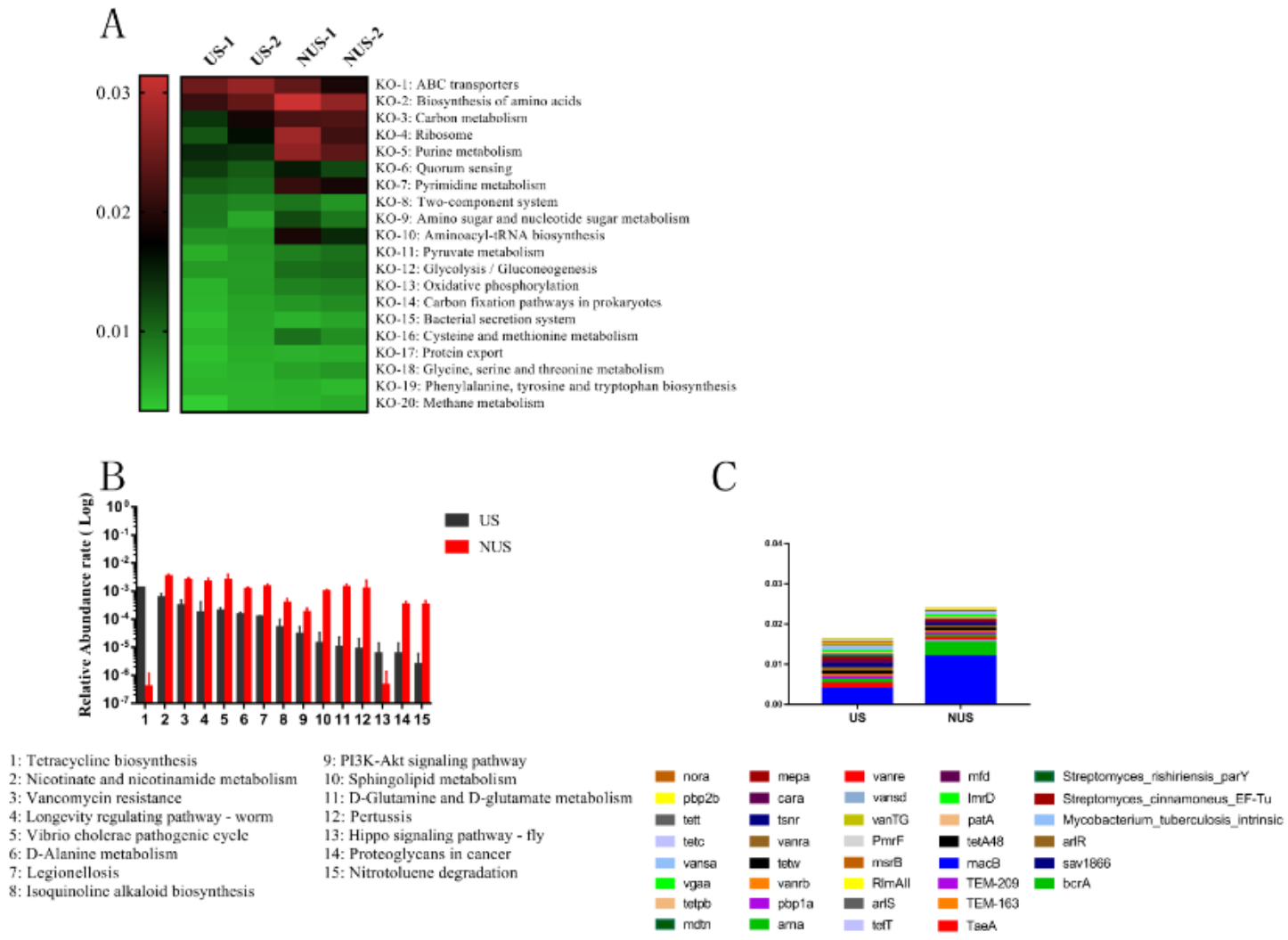

Figure 1

Deregulated metabolism of the duodenal ulcer site. A. The deregulated metabolic pathways of the ulcer site (US) than site near ulcer (NUS). The heatmap showed the altered central metabolic pathways. B. The top 15 most significantly changed pathways. C. The differential abundance of antibiotics resistance genes between the ulcer site (US) and site near ulcer (NUS). “US”：ulcer site; “NUS”：site near ulcer.

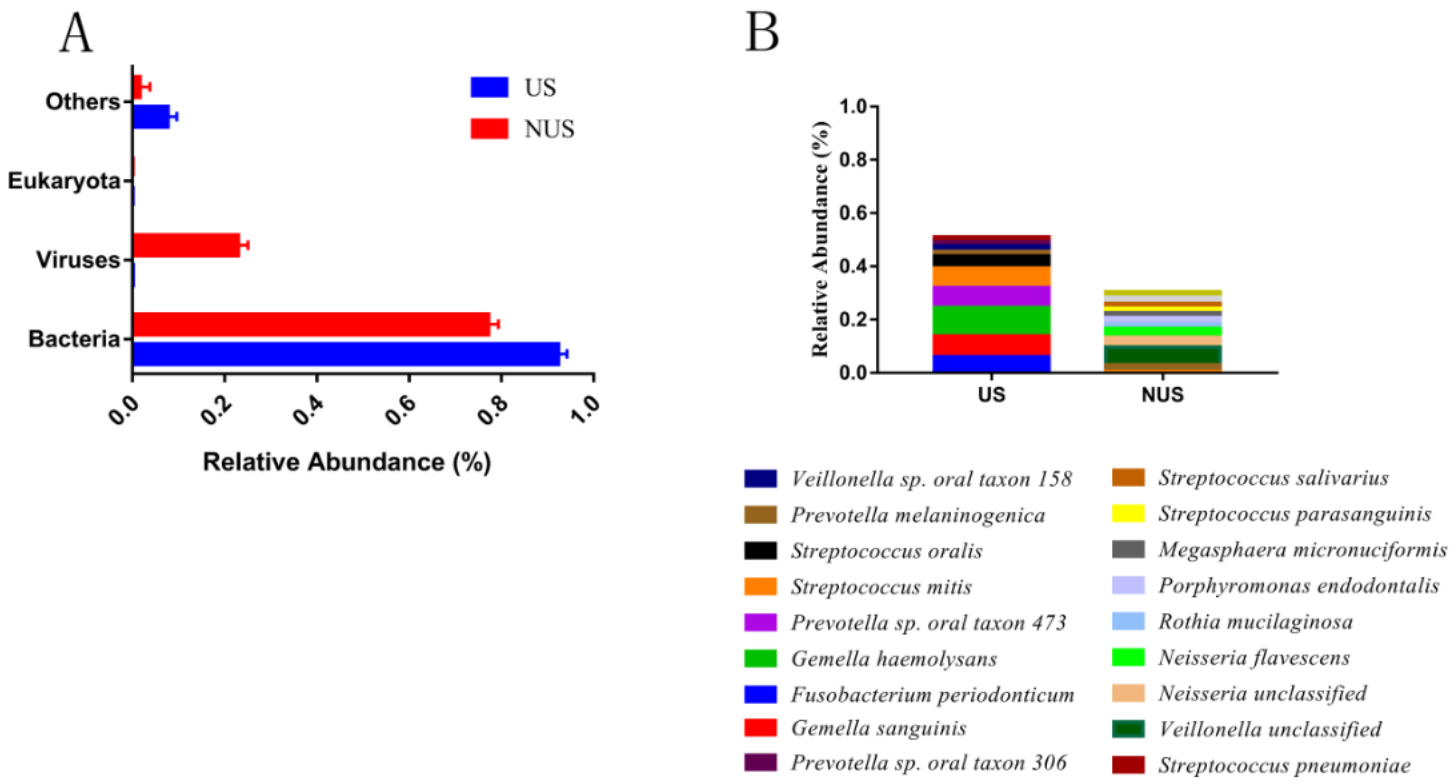

Figure 2

The microbiota difference between the duodenal ulcer site (US) and site near ulcer (NUS). A. The relative abundance difference of eukaryote, virus, bacteria and others at the duodenal ulcer site (US) and site near ulcer (NUS); B. The abundance of top 10 differential bacteria between the duodenal ulcer site and health control. “US”：ulcer site; “NUS”：site near ulcer.

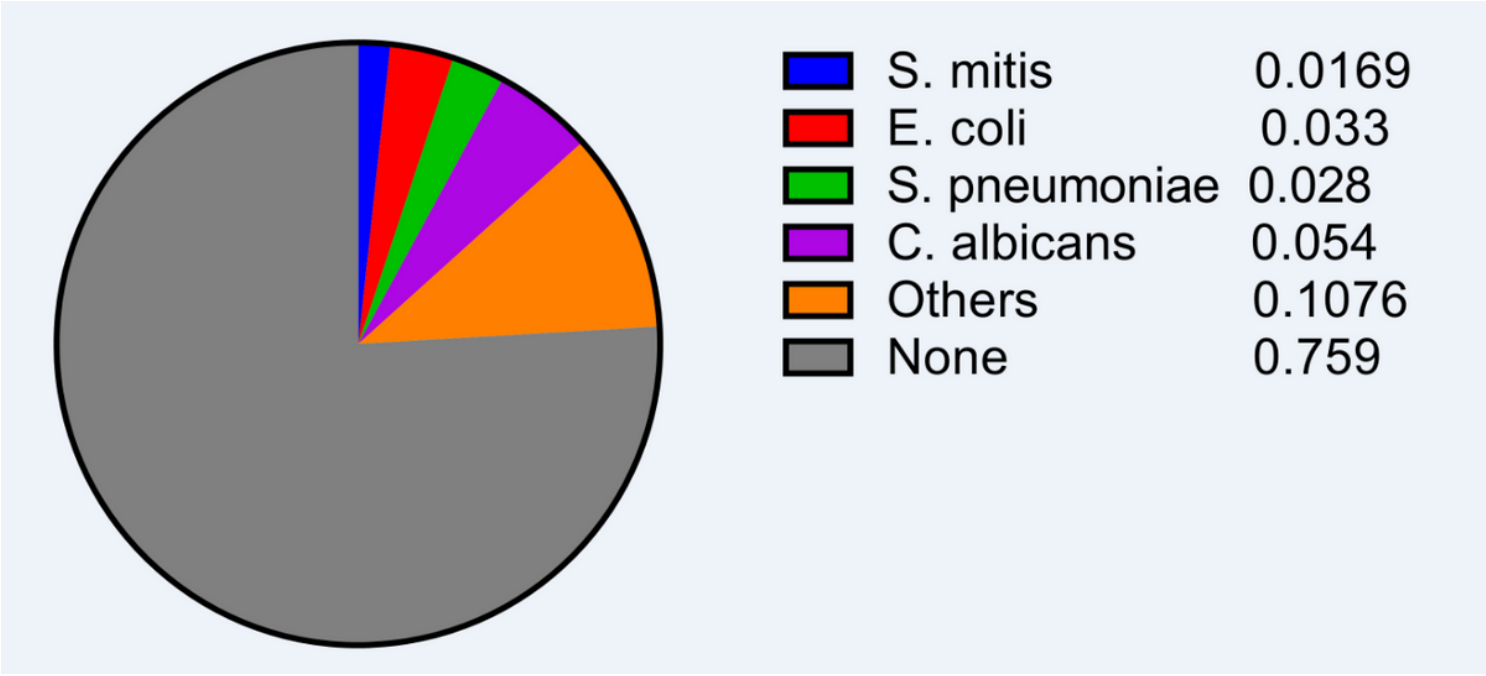

Figure 3

Microbes ratio of 353 duodenal ulcer patients cultured on agar.