

Effect of Periapillary Diverticulum on Biliary Flora and the Formation of Common Bile Duct Stone

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Effect of periampullary diverticulum on biliary flora and the formation of common bile duct stone

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Abstract

Background: Bile duct stone is closely related to periampullary diverticulum, but it is not clear whether the formation of it was affected by the diverticulum through the biliary flora. To explore the diversity and correlation of biliary and intestinal flora in the patients with choledocholithiasis and the effects of periampullary diverticulum on the flora and bile duct stone.

Methods: Bile and intestinal fluid were collected from patients with primary common bile duct stones, and then divided into diverticulum group and none-diverticulum group according to the presence or absence of paravertebral diverticula, DNA of these samples was extracted and a bacterial gene library was constructed, and related bioinformatics analysis was performed after high-throughput sequencing to obtain the bacterial components and community structure of the sample.

Result: A total of 3001,613 valid sequences were obtained, with an average of 136436.95 ± 3696.842 sequences, which were classified into 6021 ASV/OUT. Alpha diversity analysis showed that the species richness and diversity in the diverticulum group were lower than those in the non-diverticulum group. According to the species annotation results, the advantage bacterium group of the bile is Proteobacteria (BG 80.41%, Bg 70.95%), and advantage bacterium group of the intestinal fluid is Firmicutes and Proteobacteria (BG 89.39%, Bg 74.11%). A large proportion of Enterobacter was found in bile. Escherichia coli, Klebsiella,

Streptococcus and other bacteria closely related to stone formation have been found. The proportion of E. coli in the diverticulum group was increased and due to the existence of the diverticulum Enterobacteria in the bile were increased and more complex. The bacteria that produce Beta-glucuronidase are found to be increased in bile. Due to the influence of the periampullary diverticulum, the intestinal flora will be changed and then the biliary flora will also change.

Conclusion: The existence of periampullary diverticulum will affect the biliary tract flora and lead to the increase of bacteria related to stone formation, which will affect the formation of choledocholithiasis and make it easier for choledocholithiasis to form.

Keywords: primary common bile duct stones, periampullary diverticulum, biliary bacteria, intestinal bacteria, high-throughput sequencing

Background

There are thousands of species and trillions of bacteria in the human gut, which some scholars regard as the body's "hidden organs". The research found that human health is not only related to its genetic genes but also has a subtle connection with intestinal microorganism[1], gene sequencing analysis has revealed the intestinal bacteria that play an incredible role, they have important effects on health, especially the gut microbes are associated with a variety of diseases, such as cardiovascular disease, intestinal disease, autoimmune disease, metabolic disease, biliary tract disease, depression, Alzheimer's, cancer[2-9], etc. Moreover, the duodenal flora has been shown to affect the overgrowth of intestinal bacteria, irritable bowel syndrome, and celiac disease[10], and these association studies have redefined the concept of the human body as a superorganism composed of its cells and symbiotic microorganisms. Current studies have found that intestinal microbial changes can occur in

patients with primary cholangitis, but the application of ursodeoxycholic acid treatment can partially recover[11].

Common bile duct stones are a common and frequently-occurring disease, and the primary common bile duct stones are mostly pigmented stones. Increasing evidence suggests that bacteria play an important role in the pathogenesis and formation of pigmented stones[12-14]. However, only 1% of bacteria in complex samples can be cultured, and studies have shown that even if bacteria are not cultured in patients' bile, bacterial biofilms may still exist on the surface of stone[15]. This provides us with a biased view of the relative abundance of existing species.

Periampullary diverticulum refers to the saclike process formed within a radius of about 3cm around the large duodenal nipple. At present, the existence of periampullary diverticulum and choledocholithiasis, cholangitis, and choledochal dilatation is considered to be significantly related[16]. Other studies have shown that patients with common bile duct stones are more likely to be found combined with periampullary diverticulum and that the periampullary diverticulum is only related to common bile duct stones but not to gallbladder stones[17]. The existence of parapapillary diverticulum will not only cause cholestasis but also make duodenal fluid retrograde into the bile duct so that some bacteria that can produce β -glucuronidase can be infected[18]. It has been reported that even the presence of parapapillary diverticulum can lead to recurrent bacterial cholangitis[19]. Previous studies have shown that the existence of periampullary diverticulum is associated with an increased incidence of choledocholithiasis. Whether the periampullary diverticulum makes the formation of stones easier by affecting the flora is an unsolved problem at present. This study takes the flora as the starting point to explore the effect of periampullary diverticulum on choledocholithiasis.

With the emergence of a new generation of sequencing technology, people can deeply study the microbial communities in different parts of the body, including those that are difficult to cultivate, and predict the

metabolic function of the microbial community to find its impact on human health[20]. However, there is no related research that will be periampullary diverticulum associated with bacteria to analyze its influence on common bile duct stone, has yet to someone use the gene sequencing method for further study.

Methods

Subject investigated

The subjects were selected from the patients who were admitted to the Second Hospital of Hebei Medical University and planned to undergo ERCP from February 2019 to November 2019. The eligible patients were asked to sign the conversation before the ERCP, and the consent of the patients and their families was obtained. Patient inclusion criteria: 1) Diagnosed with primary choledocholithiasis 2) With no previous history of ERCP or biliary exploration surgery 3) With no antibiotics or probiotics within 3 months 4) Without severe bacteremia or sepsis with other serious diseases 5) Without emergency ERCP within 12 hours. Patients were then divided into two groups according to the intraoperative findings, namely the diverticular group and the non-diverticular group.

The experimental protocol was established, according to the ethical guidelines of the Helsinki Declaration and was approved by the Human Ethics Committee of Research Ethics Committee of the second hospital of Hebei Medical University.

Sample collection

The duodenal fluid was collected from patients who met the inclusion criteria (duodenal fluid in the diverticulum, and duodenal fluid within 3 cm around the nipples in the diverticulum) and bile. Patients' duodenal fluid and bile were collected by duodenoscope (TJF240 / JF-260V; Olympus Optical, Tokyo, Japan). After the duodenoscopy is in place, the biopsy

channel and the lens tip should be rinsed with sterile saline about 20ml, but avoid contaminating the nipple and the surrounding area with washing saline, and then pass within 3 cm around the nipple through a sterile catheter (if there is a diverticulum in Diverticulum) extract intestinal fluid about 2-5ml, and then withdraw the sterile catheter with sterile saline again in the same way. After successful cannulation with sphincterotome, before injection of contrast agent 2-5ml bile was drawn from the common bile duct. All the specimens were placed in a sterile sputum cup and immediately stored in a -80 ° C refrigerator for subsequent bacterial DNA extraction and high-throughput sequencing analysis. Finally, 11 eligible patients were selected successively, among which 5 patients (3 males and 2 females, aged 59-84 years) were combined with periampullary diverticulum. Six patients (5 males, 1 female, aged 40-80 years old) were included in the non-diverticular group. A total of 22 samples were collected. The number of bile samples in the diverticulum group was BG12~BG16. Samples of intestinal fluid were numbered: DG12~DG16. The bile samples in the non-diverticulum group were numbered as Bg6~Bg11. Samples of intestinal fluid were numbered Dg6~Dg11.

DNA extraction

The E.Z.N.A.® DNA extraction kit was used to extract DNA according to the instructions, and then DNA was quantified by Nanodrop. Finally, the extraction quality of DNA was detected by 1.2% agarose gel electrophoresis.

16S rRNA amplicon sequencing

The 16SrRNA gene V3V4 variable region was amplified by PCR, the target fragment length was 480bp, the sequencing strategy was NovaSeq-PE250, the upstream primer was 338F(ACTCCTACGGAGGCAGCA), and the downstream primer was 806R(GGACTACHVGGTWTCTAAT).

Bioinformatics analysis process

The sequence denoising is performed according to the analysis process of QIIME2 DADA2[21]. According to the distribution of ASV/OTU in different samples, evaluate the Alpha diversity of each sample and calculate the corresponding indexes, and corresponding graphs were drawn. Species annotation was performed, and a histogram of species composition was then produced for all samples at the phylum and genus levels. GraPhlAn[22] was used to draw the GraPhlAn evolutionary tree graph to show the evolution of each sample in different species and understand the overall situation. Use the Venn diagram for community analysis to study which species are common and which are unique among different samples.

Result

Sample sequence and ASV/OTU

Sequence denoising is carried out by using the DADA2 method after sequencing. In the past, sequences with similarity higher than a certain threshold are usually merged into an OTU (OperationalTaxonomicUnit, operable taxon)[23], but the DADA2 method is no longer clustering with similarity, but only removes repetition, which is equivalent to clustering with 100% similarity. Each sequence that removes repetition is called ASV (amplicon sequence variants), or call it a feature sequence (corresponding to the OTU representative sequence). After quality control, denoising, splicing, and de-chimerism of the original sequence, we finally obtained 3501497 original sequences and 3002685 high-quality sequences. Finally, after removing the ASV sequence with a total number of only 1 in all samples by default, 3001613 valid sequences were obtained, with an average of 136436.95 ± 3696.842 (Fig 1A and 1B).

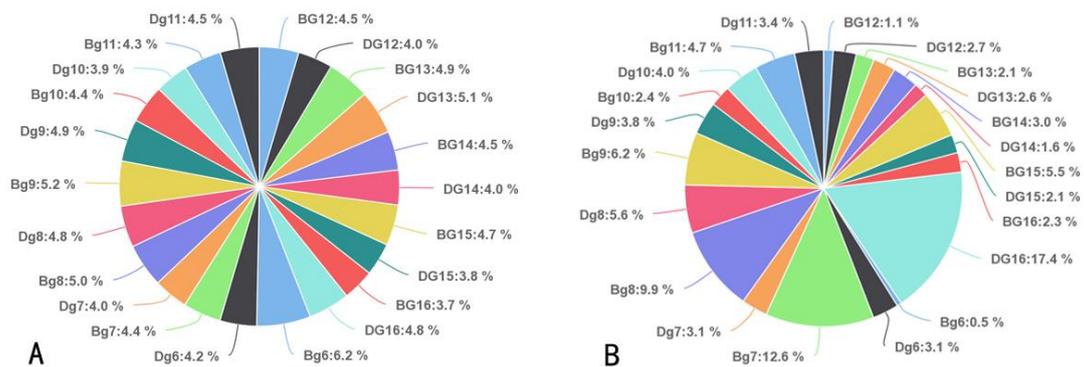


Fig.1 A Proportion of Valid Sequences per Sample .B Proportion of ASV/OUT per Sample.

Alpha diversity analysis

Alpha diversity is a comprehensive index, which can not only reflect the species diversity in the sample area, but also describe the richness and evenness Alpha diversity is evaluated by calculating the corresponding diversity index, the richness is characterized by Chao1 index and Observedspecies index, the higher the community richness is, the larger the index is, and the higher the index value is, the higher the community diversity is. The coverage is characterized by the Goodscovrage index. The higher the index, the less the proportion of undetected species in the sample. The specific results are shown in Table 1.

Table 1.Date output and ASV/OUT result

Sample	Chao1 index	Simpson index	Shannon index	Observed_species index	Goods_coverage index
BG12	344.243	0.376161	1.60925	332.7	0.999681
DG12	542.984	0.905654	4.69197	534.4	0.999616
BG13	612.447	0.9488	6.30349	603.4	0.999784
DG13	415.731	0.819593	4.00397	400.2	0.999616
BG14	547.107	0.918914	5.99519	544.4	0.99992
DG14	333.107	0.800385	3.65764	328.7	0.99981
BG15	851.382	0.957583	6.80147	845.6	0.999849
DG15	617.942	0.954548	5.77077	614.5	0.999831
BG16	477.473	0.416398	1.88606	474.9	0.999817
DG16	1554.1	0.971758	8.10305	1546.2	0.999775
Bg6	149.093	0.232982	0.720502	134.7	0.999701
Dg6	703.398	0.970911	6.56541	693	0.99978
Bg7	1326.94	0.96573	7.18555	1319.8	0.999708
Dg7	621.034	0.954163	5.95873	616.1	0.999778
Bg8	1108.56	0.950868	6.96485	1096.6	0.999722
Dg8	982.866	0.976595	6.93822	958.9	0.99948
Bg9	1226.83	0.958671	6.66904	1185.5	0.999159
Dg9	873.366	0.970639	6.97735	853.1	0.99954
Bg10	551.433	0.916973	5.71566	534.5	0.999707
Dg10	850.602	0.979503	6.96761	845.9	0.999727
Bg11	897.826	0.97065	6.92461	891.6	0.999802
Dg11	772.199	0.958219	6.45054	760.6	0.999744

Rarefaction curves and Rank abundance curve

Rarefaction curve can be used to evaluate the diversity and abundance of sample species, so we constructed the Observed species index rarefaction curve and Shannon index rarefaction curve(Fig 2, 3).

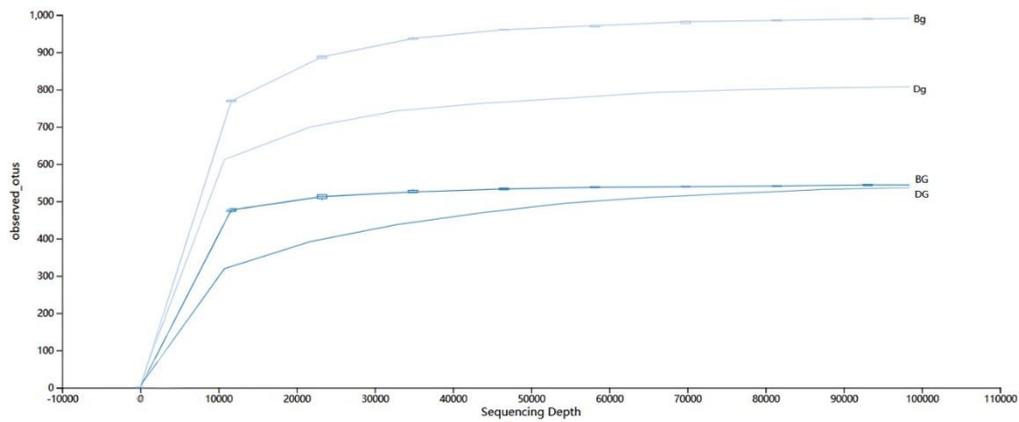


Fig.2.Observed species rarefaction curve. It can be seen from the figure that the Observed species index sparse curve of each group rises rapidly at the beginning, but with the increase of sequencing depth, the curves eventually become flat. The index value of the non-diverticulum group was higher, among which the value of the Bg group was the highest; the index value of the diverticulum group was relatively low, and the two groups of samples in the group finally tended to be one.

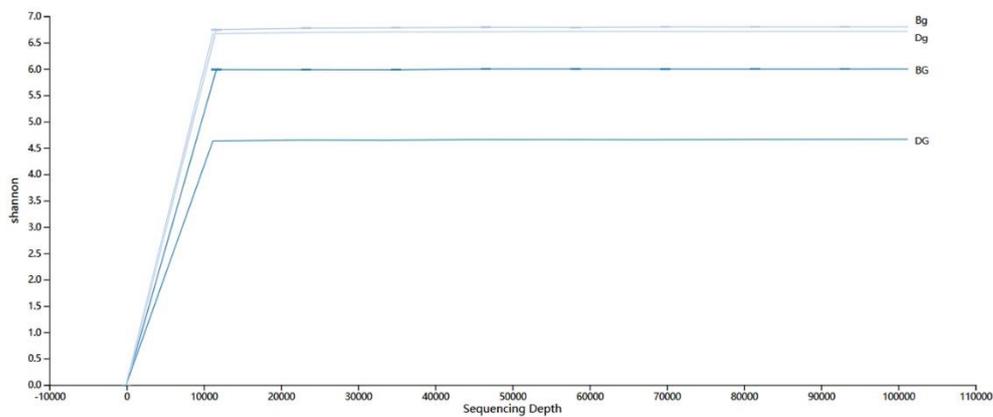


Fig.3 Shannon rarefaction curve. The samples of the non-diverticulum group are located at the upper part, and the diverticulum group is located at the lower part, among which the value of Bg Group is the highest and close to that of Dg group, indicating that the diversity of non- diverticulum group sample is relatively high and bile and intestinal fluid are similar; the values of BG Group and DG group are low, indicating that the diversity of bile sample in diverticulum group is lower and higher than that of intestinal fluid.

The rank abundance curve can be used to reflect the ASV/OUT abundance distribution of each sample (Fig 4). Different from the rarefaction curve, the rank abundance curve can reflect species diversity and the richness and uniformity of sample species. The richer the species composition, the wider the curve. The width of the curve reflected the abundance of the species. The smoother the curve, the more uniform is the species distribution. Similarly, the curve of the non-diverticular group was wider and smoother than that of the diverticular group.

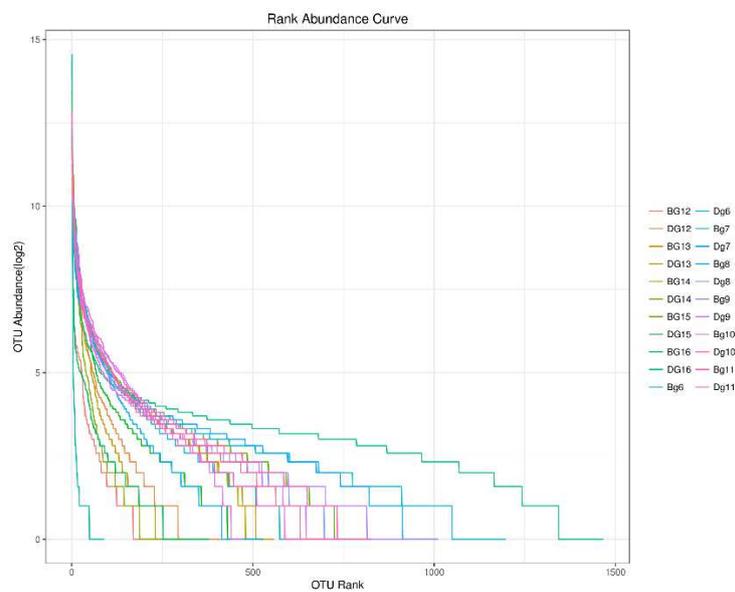


Fig.4 Rank-Abundance curve

Annotation of the taxonomy of species

Greengenes database was used to annotate each ASV/OTU feature sequence by QIIME2 (2019.4) 's classify-sklearn algorithm, and the samples were annotated from seven taxa including kingdom, phylum, class, order, family, genus, and species (Fig 5).

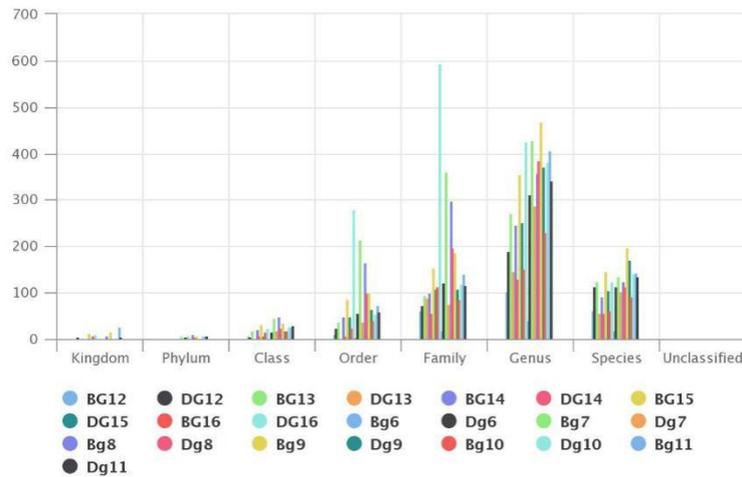


Fig.5 Distribution of sequences per taxonomic level per sample. Statistics of the number of sequences in each taxon level can be used to evaluate the species annotation resolution of each sample. The higher the proportion of annotation to genus and species, the better the annotation effect. Through Figure 6, we can see intuitively that the proportion of each sample at the genus and species level is high, especially at the genus level, and the proportion at the gate level is low, which indicates that the species annotation resolution of annotation results is high, and it also provides a good database for subsequent analysis.

Phyla-level bacteria are relatively conservative and can reflect the differences and changes of colonized flora in the same place among different people(Fig 6), Proteobacteria (BG 80.41% vs. BG 70.95%) were the most abundant in the bile samples of 11 patients, followed by Bacteroidetes (BG 7.26% vs. BG 11.23%), Firmicutes (BG 7.15% vs. BG 7.86%) and Actinobacteria (BG 2.19% vs. BG 4.73%). The species distribution and abundance of the two groups were similar in phylum classification.

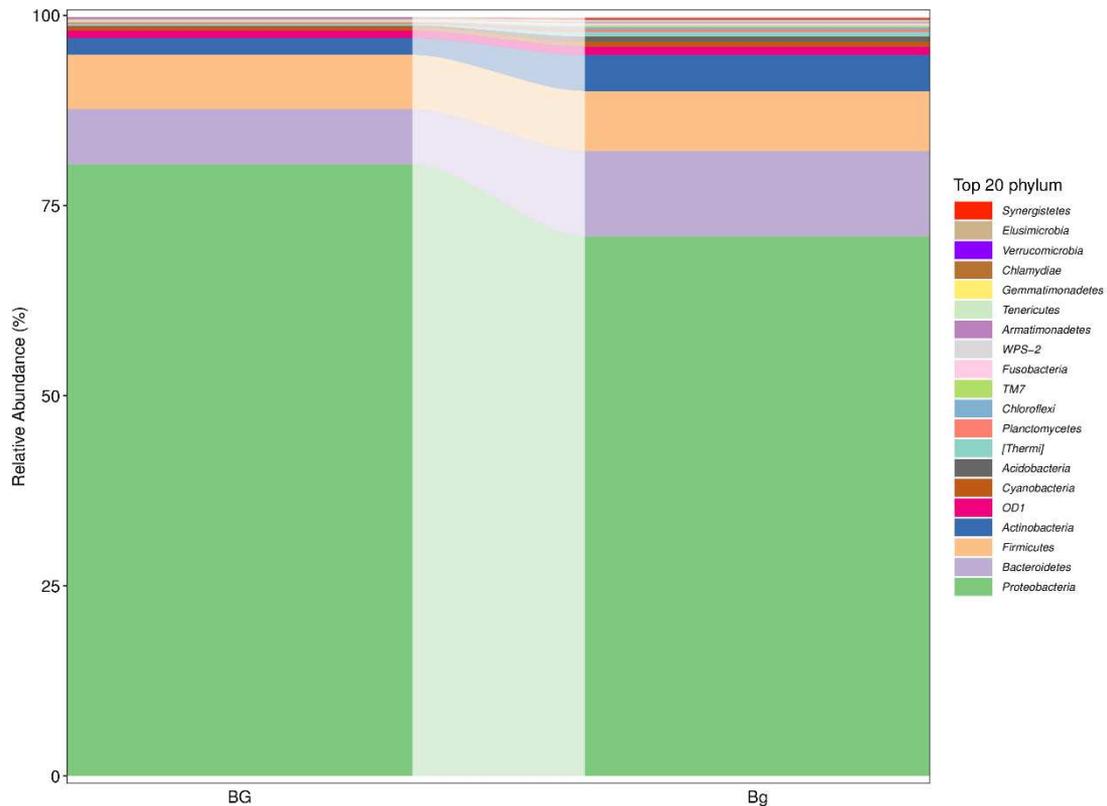


Fig.6 TOP20 species distribution at phylum level of all bile samples

Among the 11 intestinal fluid samples(Fig 7), Firmicutes (DG 51.35% vs. DG 28.64%) had the highest abundance in the diverticulum group, while Proteobacteria (DG 38.04% vs. DG 45.47%) had the highest abundance in non-diverticulum intestinal fluid. Actinobacteria (DG 5.68% vs DG 7.87%) and Bacteroidetes (DG 2.78% vs DG 9.22%) were the next most common bacteria. The distribution of intestinal fluid in diverticulum is similar to that of non-diverticular intestinal fluid phylum, but the abundance ratio is different, indicating that the existence of diverticulum may change the distribution proportion of bacteria in the intestinal diverticulum. Proteobacteria, Bacteroides, Firmicutes, and Actinobacteria were the most important bacteria in each sample. This distribution of bacteria in the intestinal tract reported in the relevant literature of the flora is consistent. However, the proportion of Proteobacteria in the intestinal flora of patients with common bile duct stones is abnormally high, and the change in the proportion of bacteria may be related to the formation of stones.

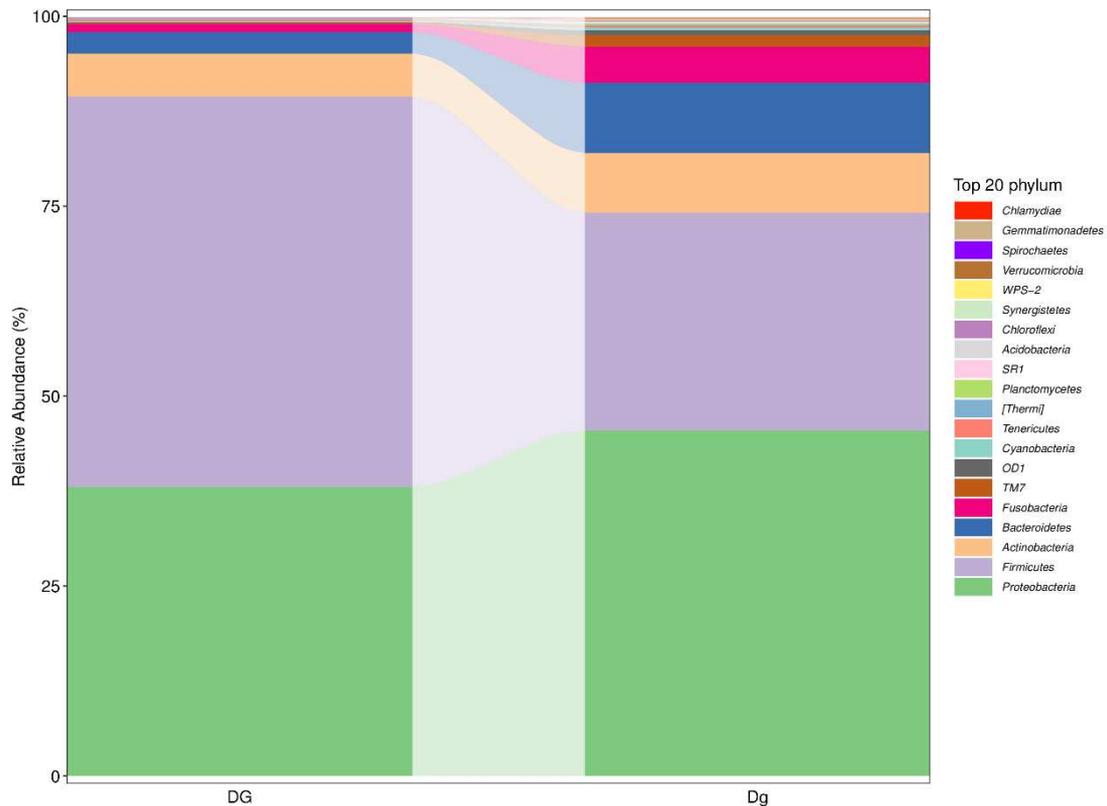


Fig.7 TOP20 species distribution at phylum level of all Intestinal samples

The general composition of the genus can reflect the change of habitat in different parts of the human body(Fig 8, 9). Genus is the lowest taxon except for species in the bacterial taxon. However, due to the wide variety of microorganisms and the lack of accurate species information in the current database, the microbial sequences have not been completely covered by the sequencing sequence or the reference sequence lacks accurate species information, leading to the inability to identify unclassified species among some specific genera. Therefore, some characteristic sequences may not be able to obtain genera-level annotation information in the analysis process.

The highest proportion of the bile samples in the diverticulum group was unclassified_Enterobacteriaceae (BG 32.19%), which could not be classified and not included in the figure. Besides, the highest proportion was found was Ochrobactrum (BG 12.77%), and then were Sediminibacterium (BG 6.46%), Escherichia (BG 4.74%), Cupriavidus (BG 3.36%), Veillonella (BG 2.84%), Acinetobacter. (BG 2.33%), Agrobacterium (BG 2.14), Sphingomonas (BG 2.05%), Pseudomonas (BG 1.14%), etc. In the bile samples of the non-diverticulum group, the highest proportion of microflora was Enterobacter (Bg 16.30%), followed by Ochrobactrum,(Bg 13.59%), Sediminibacterium,(Bg 8.66%), Cupriavidus (Bg 4.36%), Anoxybacillu (Bg 3.27%), Acinetobacter (Bg 2.80%), Sphingomonas (Bg 2.66%), Agrobacterium (Bg 2.31), Tepidimonas (Bg 2.20%), etc.

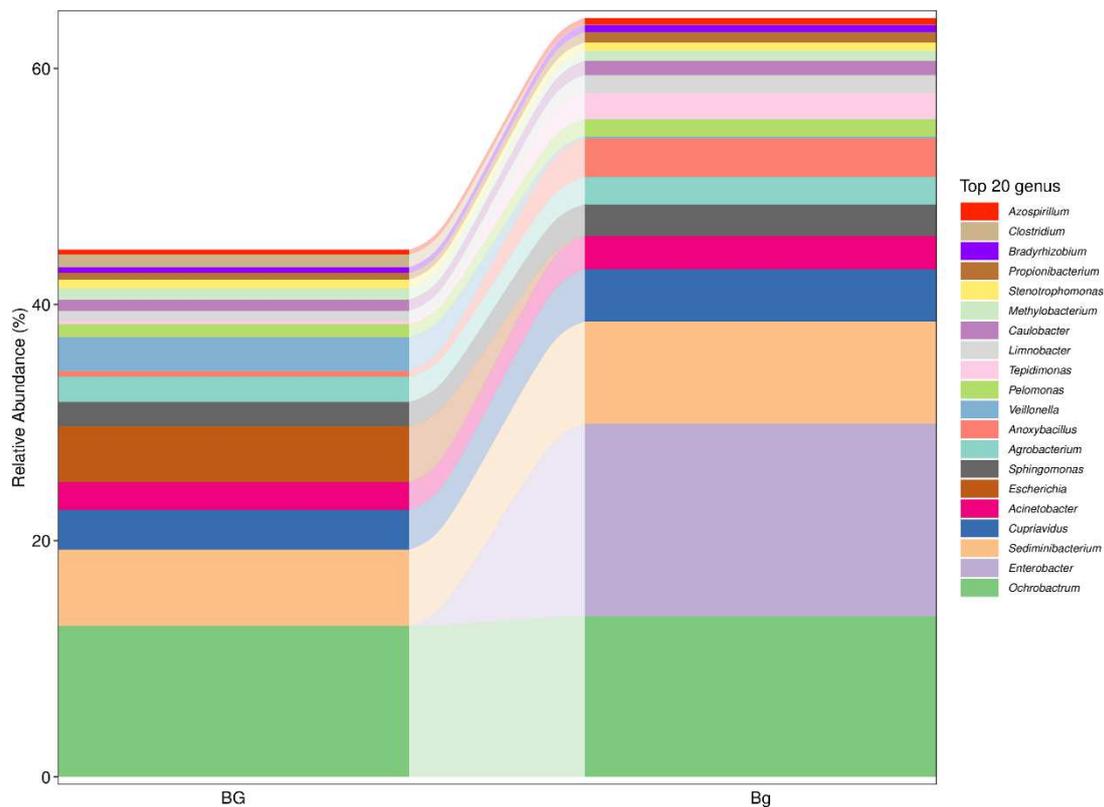


Fig 8. TOP20 species distribution at genus level of all bile samples

From S8 Fig, it can be seen that Enterobacter is the most different between the two groups, there is no Enterobacter in the BG group. This is due to technical limitations not able to obtain genera-level annotation information in the analysis process. But its Enterobacteriaceae do occupy a relatively high proportion, indicating that the proportion of Enterobacter in the bile of the diverticulum group is higher and more complicated, and its analysis needs further exploration. It can also be seen from Figure 9 that there is a certain proportion of Escherichia in the bile of the diverticulum group, which is not found in the bile of the non-diverticulum group. Secondly, Veillonella also has a higher share in BG than in the Bg group. Proportion, the rest of the genus can be seen from the chart that the two groups are not much different.

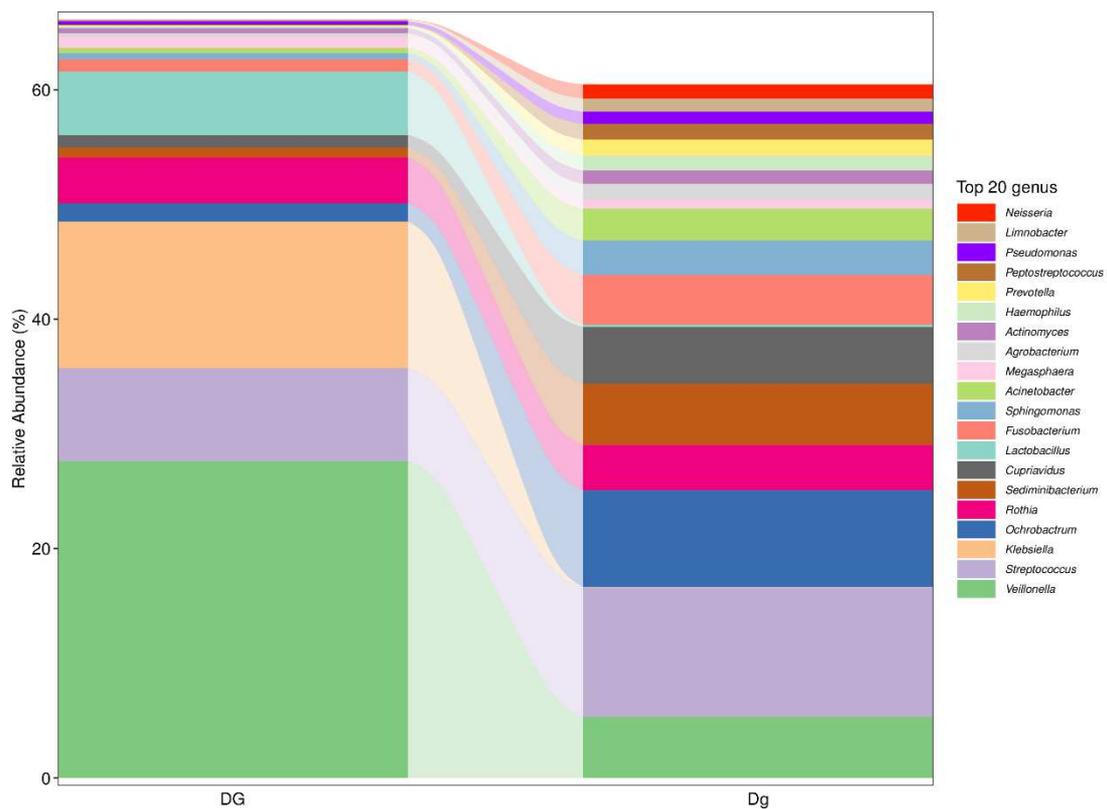


Fig 9. TOP20 species distribution at genus level of all Intestinal samples

S9 Fig shows the distribution of TOP20 species in the genus level in all intestinal fluid samples. The figure also shows the 20 species with the

highest proportions except those that cannot be classified. Among the intestinal fluid samples of the diverticulum group, the highest proportion of flora was *Veillonella* (DG 27.63%), followed by unclassified *Enterobacteriaceae* (15.71%), because it has not been classified yet draw into the picture, then *Klebsiella* (DG 12.75%), *Streptococcus* (DG 8.12%), *Lactobacillus* (DG 5.56%), *Rothia* (DG 4.00 %), *Ochrobactrum* (DG 1.58%), *Cupriavidus* (DG 1.07%), *Fusobacterium* (DG 1.05%), etc. Among the intestinal fluid samples from the non-diverticulum group, the highest proportion of flora was *Streptococcus* (Dg 11.28%), followed by *Ochrobactrum* (Dg 8.41%), *Sediminibacterium* (Dg 5.36%), *Veillonella* (Dg 5.35%), *Cupriavidus* (Dg 4.93%), *Fusobacterium* (Dg 4.34%), *Rothia* (Dthia 3.94%), *Sphingomonas* (*Sphingomonas*, Dg 3.01%), *Acinetobacter* (Dg 2.76%), *Prevotella* (Dg 1.39%), etc. Expectedly, both *Enterobacter* and *Streptococcus* accounted for a high proportion in the intestinal fluid samples, but through Figure 10 we can find that the two groups with a large difference in graphics are firstly *Veillonella*, which has a higher proportion in DG. (27.63%), while the proportion of Dg is relatively small (5.35%), and in the BG group, *Weirongococcus* is also found to occupy a certain proportion and is higher than the Bg group. It can also be seen from the figure that the DG group is Cray The genus *Pleurotus* has a high proportion (12.75%), while it is almost absent in the Dg group (0.03%). Its proportion in the BG group is 0.26%, but it is also almost absent in the Bg group (0.01%). The proportion of the DG group (5.56%) is also much higher than that of the Dg group (0.19%). This is no accident, indicating that the existence of diverticulum not only changes its flora but also affects the biliary flora. *Propionibacterium* is a typical bacterium in the formation of cholelithiasis. Currently, there are few reports on the isolation of *Propionibacterium* from bile, which is only isolated and found in a single study. However, *Propionibacterium* is found in all the samples in this study, BG 0.55%, Bg0.84%, DG0.08%, Dg0.39%.

Species Difference Analysis and Marker Species

There are 626 ASV/OTUs shared by the BG and Bg group, and the species abundance composition of their consensus characteristic sequences at the phylum and genus levels is shown in S10 Fig. BG and Bg shared species abundance in the order of Proteobacteria, Bacteroidetes, Firmicutes, and Actinobacteria at the phylum; Ochrobactrum, Enterobacter, Sediminibacterium, Cupriavidus, Sphingomonas, Acinetobacter, Anoxybacillus, Escherichia, Clostridium, and Veillonella at the genus.

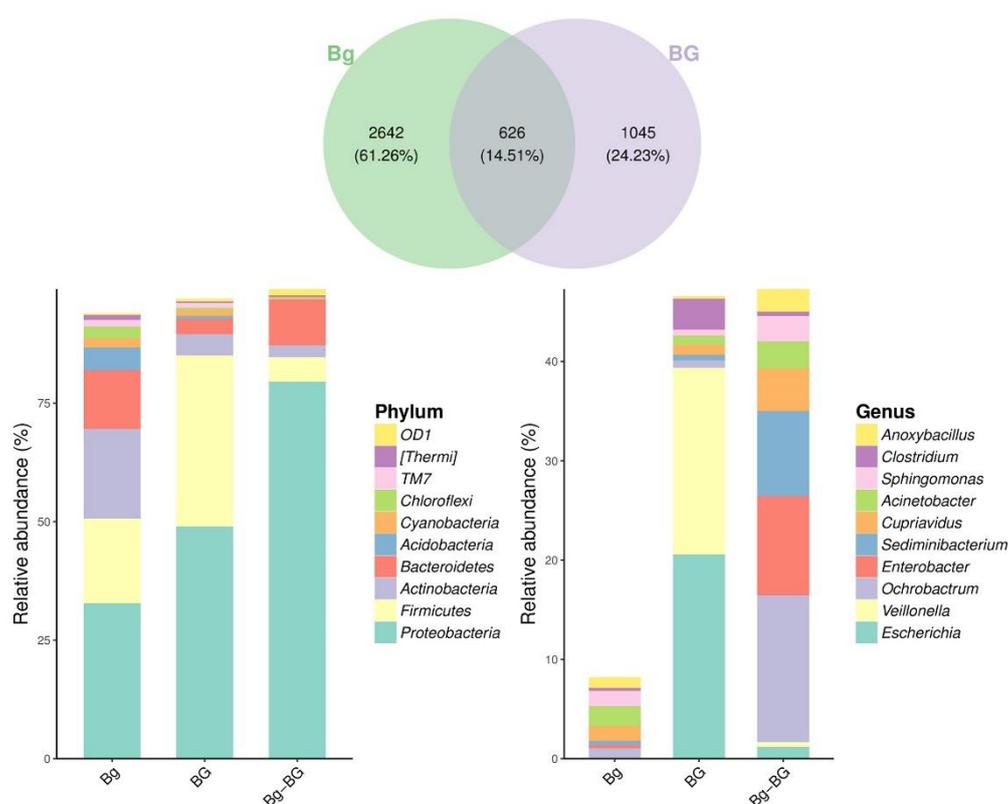
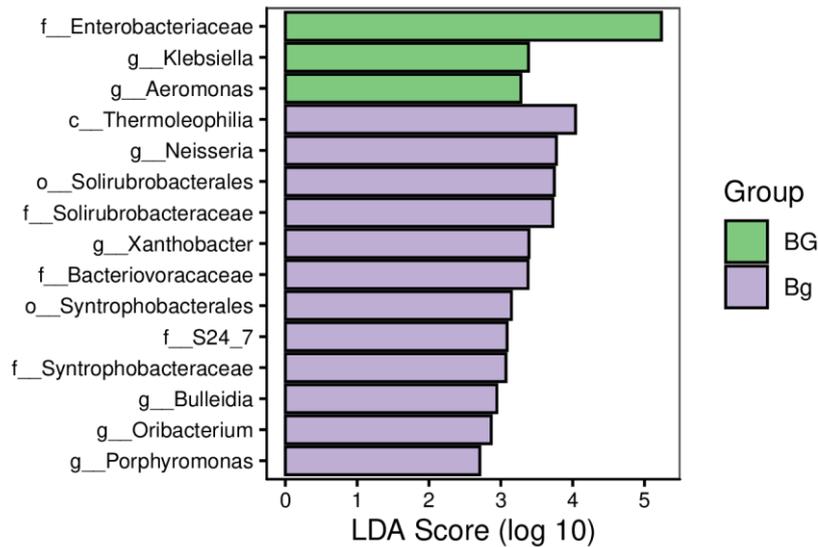


Fig.10 Venn diagram of BG and Bg groups and ASV/OTU abundance

Lefse analysis is an analysis method to find species with obvious differences between groups. It can directly analyze the differences of all classification levels at the same time, to analyze the marker species of each group. S11 Fig is the histogram of the LDA effect value of the marker species generated by this method. It can be seen from the graph that the

species difference between BG and Bg is obvious. Enterobacteriaceae,



Klebsiella, and Aeromonas are unique in the BG Group.

histograms of common sequences at phylum and genus level

Fig.11 The bar chart of LDA effect value of marker species

Discussions

As one of the common diseases in choledocholithiasis, the mechanism of primary choledocholithiasis has not been fully elucidated, but most of them believe that bacterial infection plays an important role in the formation of choledocholithiasis. Bacterial infection and abnormal bilirubin metabolism will cause calcium bilirubin to deposit and accumulate into nucleation in the biliary tract, resulting in the formation of stones, especially the formation of pigment stones is more thought to be caused by a bacterial infection. Through the study of people without biliary diseases, it has been proved that the biliary tract is sterile under normal conditions[24], while bacteria grow in the bile culture of almost all patients with choledocholithiasis. And all bile samples in this study were also detected by high-throughput sequencing.

Our study found that the biliary tract of choledocholithiasis patients possesses as abundant microbial colonies as the intestine. Four main types of bacteria were found in all the samples in this study: Firmicutes,

Proteobacteria, Actinobacteria, and Bacteroidetes, which accounted for more than 95% of the samples in each group, of which Proteobacteria was the dominant bacteria in bile samples, and the dominant bacteria in intestinal fluid samples were Firmicutes and Proteobacteria. According to relevant literature reports, it was found that the dominant bacteria in the human intestinal flora were Firmicutes and Bacteroidetes[25, 26], which accounted for more than 90%, while in patients with choledocholithiasis, the proportion of Proteobacteria increased excessively, while the proportion of Bacteroidetes decreased relatively. Another study on patients with gallstones also found that there were a large number of bacteria in the bile duct, and there was excessive growth of Proteobacteria in the intestinal flora[27]. It shows that there is an intestinal flora imbalance in patients with choledocholithiasis. The higher proportion of Proteobacteria in the bile, which is very similar to that of the duodenum[28], suggests that these bacteria are more from retrograde infection of the duodenum.

Proteus contains many well-known pathogenic bacteria, such as Escherichia coli, Helicobacter pylori, Salmonella, Vibrio cholera, etc., especially Escherichia coli is closely related to stone formation. This study also confirmed that there are a large number of Escherichia coli and other Enterobacter in bile. It can be seen that the increase of Proteobacteria is closely related to the formation of stones, and it also predicts that these patients will also have an increased risk of other intestinal diseases. The bile flora is not only very similar to the intestinal fluid flora around the duodenal papilla but also changes when the intestinal flora changes due to the existence of diverticulum. Bacterial infection through the blood pathway generally causes chills, fever and other symptoms, but all the patients in this group have no fever before the operation, and there is no significant increase in white blood cells in the blood routine, so it can be seen that the possibility of bacterial infection through the blood pathway is relatively small. It has also been reported that bacteria have been cultured in the bile of asymptomatic patients, indicating that this is not accidental.

Thus it can be seen that the bacteria in bile may be more derived from the intestinal tract, more specifically from around the duodenal papilla.

It is well known that bile is a natural antibacterial compound and essential for digestion and nutritional absorption, but some enteropathogens have not only evolved to resist the bactericidal conditions of bile, but these bacteria also use bile as a signal to enhance virulence regulation and thus effectively infect, and *E. coli* is one of them[29]. At present, it is believed that the mechanism of stone formation caused by bacteria is mainly related to several factors or enzymes produced by bacteria, such as β -glucuronidase, phospholipase, and bacterial hydrolase. This study also found that the existence of diverticulum can increase the production of β -glucuronidase species in bile. β -glucuronidase is a key enzyme that regulates the release of free bilirubin and glucuronic acid from bilirubin glucuronic acid. Free bilirubin precipitates with free calcium ions to generate calcium bilirubin, which is the main component of pigmented stones. Calcium palmitate and fatty acids in pigmented stones (accounting for 10-20% of the content of brown pigmented stones) are related to bacterial phospholipase activity. *Escherichia coli*, *Enterococcus*, *Klebsiella*, *Acinetobacter*, and *Streptococcus* have β -glucuronidase activity, which is more common in pigmented stones, and *Klebsiella* is positively correlated with serum bilirubin levels a large proportion of these bacteria appeared in the bile of the diverticulum group, indicating that the presence of diverticulum increased the number of bacteria related to the formation of pigmented stones in bile and made the formation of pigmented stones easier.

Lactobacillus is a kind of probiotics, but it is easy to retrograde into the bile duct because of its over-reproduction due to the relatively closed environment formed by diverticulum. In this study, we found that *Lactobacillus* was rarely found in the bile of the non-diverticulum group, while they did appear in the bile of the diverticulum group. *Lactobacillus* has bile acid hydrolase activity, and the decrease of bile acid concentration

plays an important role in the formation of pigmented stones. Its existence is bound to have a certain impact on the formation of stones. Also, a large proportion of *Veillonella* was found in bile and intestinal fluid in the diverticulum group. At present, *Veillonella* is associated with primary sclerosing cholangitis and Crohn's disease. Concerning *Helicobacter pylori*, it has been reported that it is related to the formation of gallstones, but it has not been found in the bile of patients in this study, it may not have much to do with the formation of pigmented stones.

In this study, gene sequencing was used to sequence the bile and intestinal fluid of patients with choledocholithiasis. To eliminate the interference of various reasons to ensure the singleness of variable factors, especially the influence of antibiotics, it is relatively difficult to obtain samples. Fewer patients can be sampled, and the number of samples is less, which is also the deficiency of this study. Therefore, to further explain the relationship between the formation of choledocholithiasis and the microenvironment of bile duct flora, and find more research clues, we need to continue to collect samples and expand the sample size. At the same time, it has been reported that bacteria exist in all layers of pigmented stones, especially calcium layers, indicating that they are related to the early stage of stone formation and affect the precipitation of calcium bilirubin in the process of bacterial unconjugation. Due to the limitations of the methods and techniques of this study, it has not been proved that the effect of bacteria on stones is the initial dynamic factor or involved in the whole process, and the relevant bacterial products have not been detected. It can only be speculated from the relevant literature reports. Intestinal and biliary tract bacteria are closely related to the formation of choledocholithiasis. A more in-depth study is needed to explain the formation mechanism of choledocholithiasis from the perspective of microflora.

Conclusion

The existence of periampullary diverticulum will affect the biliary tract flora and lead to the increase of bacteria related to stone formation, which will affect the formation of choledocholithiasis and make it easier for choledocholithiasis to form.

Declarations

Abbreviations

JPDD Juxtapapillary duodenal diverticulum

ERCP Endoscopic retrograde cholangiopancreatography

PCR Polymerase Chain Reaction

ASV Amplicon sequence variants

OTU Operational Taxonomic Unit

LDA Linear discriminant analysis

LEfSe LDA Effect Size

Ethics approval and consent to participate

The experimental protocol was established, according to the ethical guidelines of the Helsinki Declaration and was approved by the Human Ethics Committee of Research Ethics Committee of the second hospital of Hebei Medical University. Written informed consent was obtained from individual or guardian participants.

Consent for publication

Not applicable

Availability of data and material

All data generated or analyzed during this study are included in this published article.

Competing interests

The authors declare that they have no competing interests.

Funding

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Authors' contributions

SH* contributed to the conception of the study;

TY performed the experiment;

LZ contributed significantly to analysis and manuscript preparation;

HW performed the data analyses and wrote the manuscript;

ZQ helped perform the analysis with constructive discussions.

All authors have read and approved the manuscript, and ensure that this is the case.

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Figures

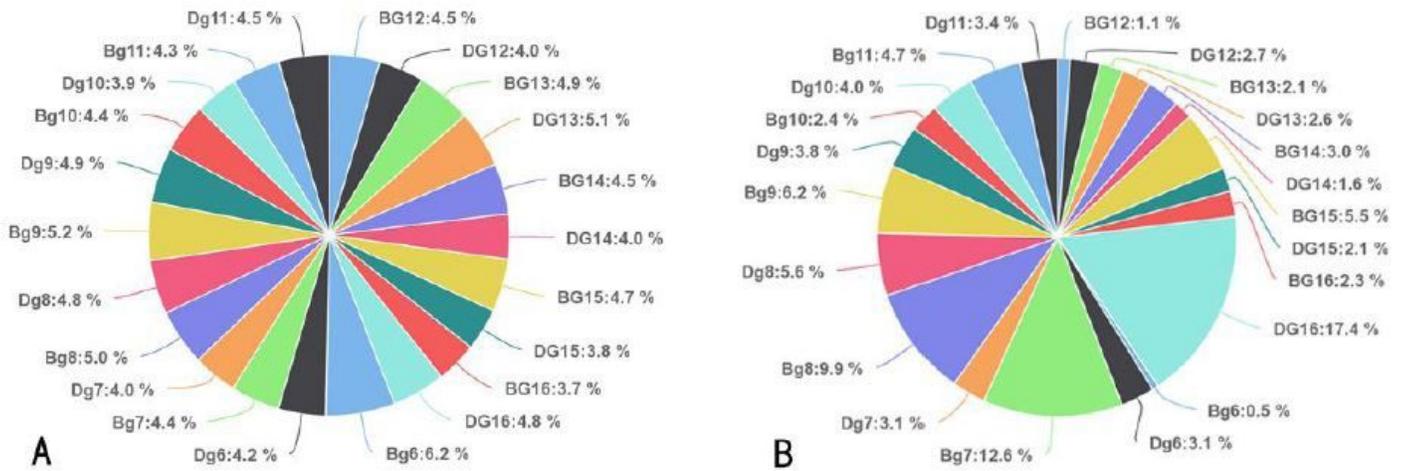


Figure 1

A Proportion of Valid Sequences per Sample .B Proportion of ASV/OUT per Sample.

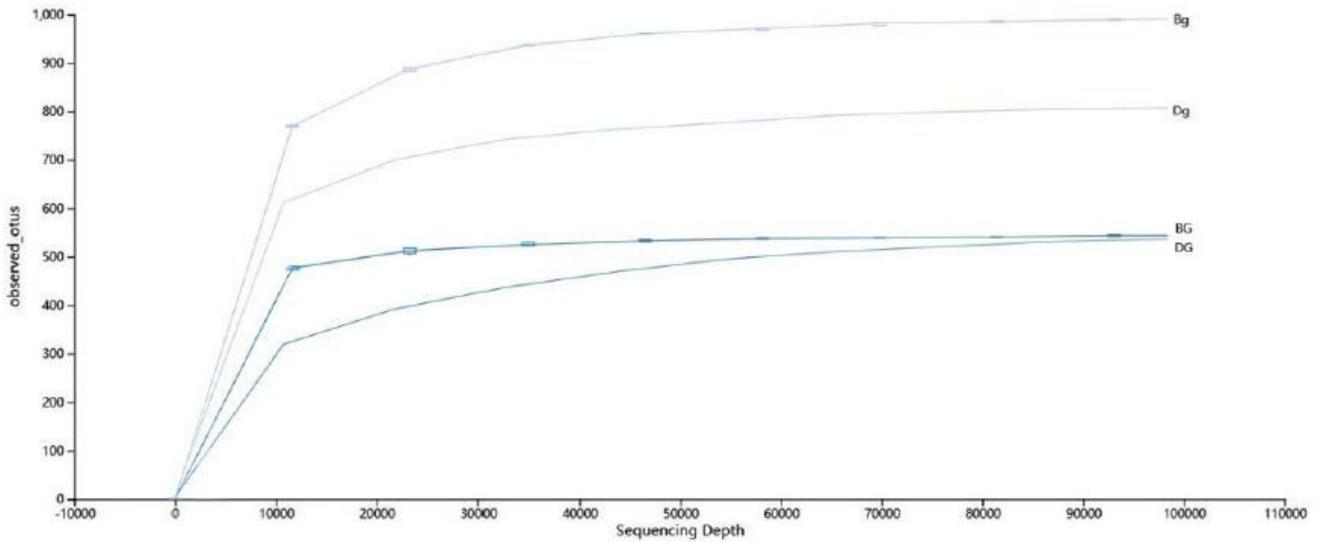


Figure 2

Observed species rarefaction curve. It can be seen from the figure that the Observed species index sparse curve of each group rises rapidly at the beginning, but with the increase of sequencing depth, the curves eventually become flat. The index value of the non-diverticulum group was higher, among which the value of the Bg group was the highest; the index value of the diverticulum group was relatively low, and the two groups of samples in the group finally tended to be one.

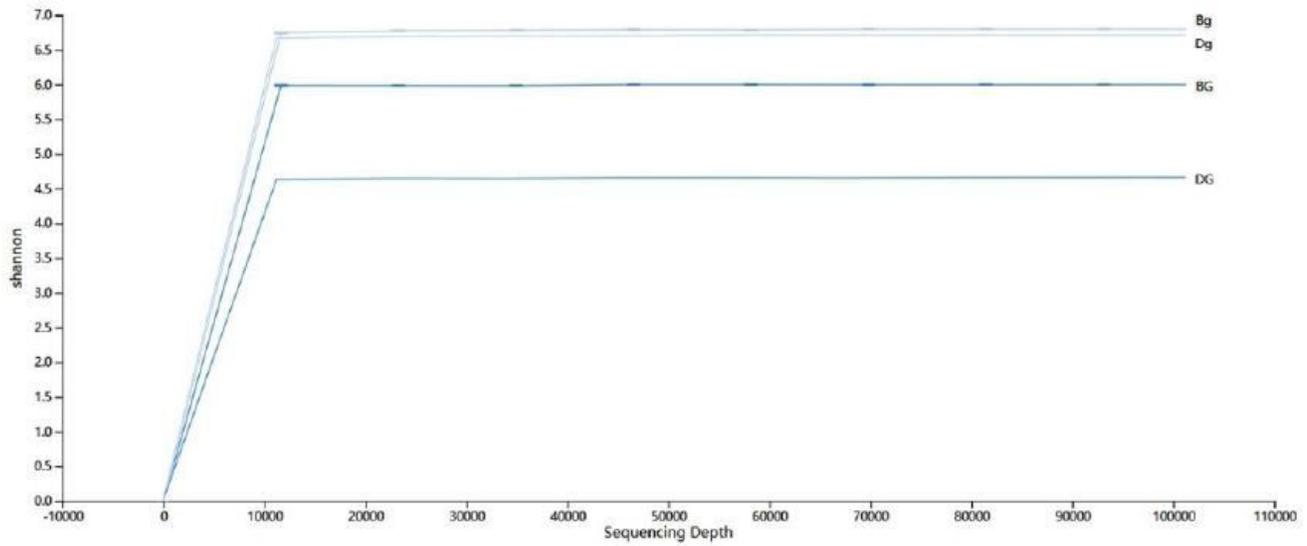


Figure 3

Shannon rarefaction curve. The samples of the non-diverticulum group are located at the upper part, and the diverticulum group is located at the lower part, among which the value of Bg Group is the highest and close to that of Dg group, indicating that the diversity of non-diverticulum group sample is relatively high and bile and intestinal fluid are similar; the values of BG Group and DG group are low, indicating that the diversity of bile sample in diverticulum group is lower and higher than that of intestinal fluid.

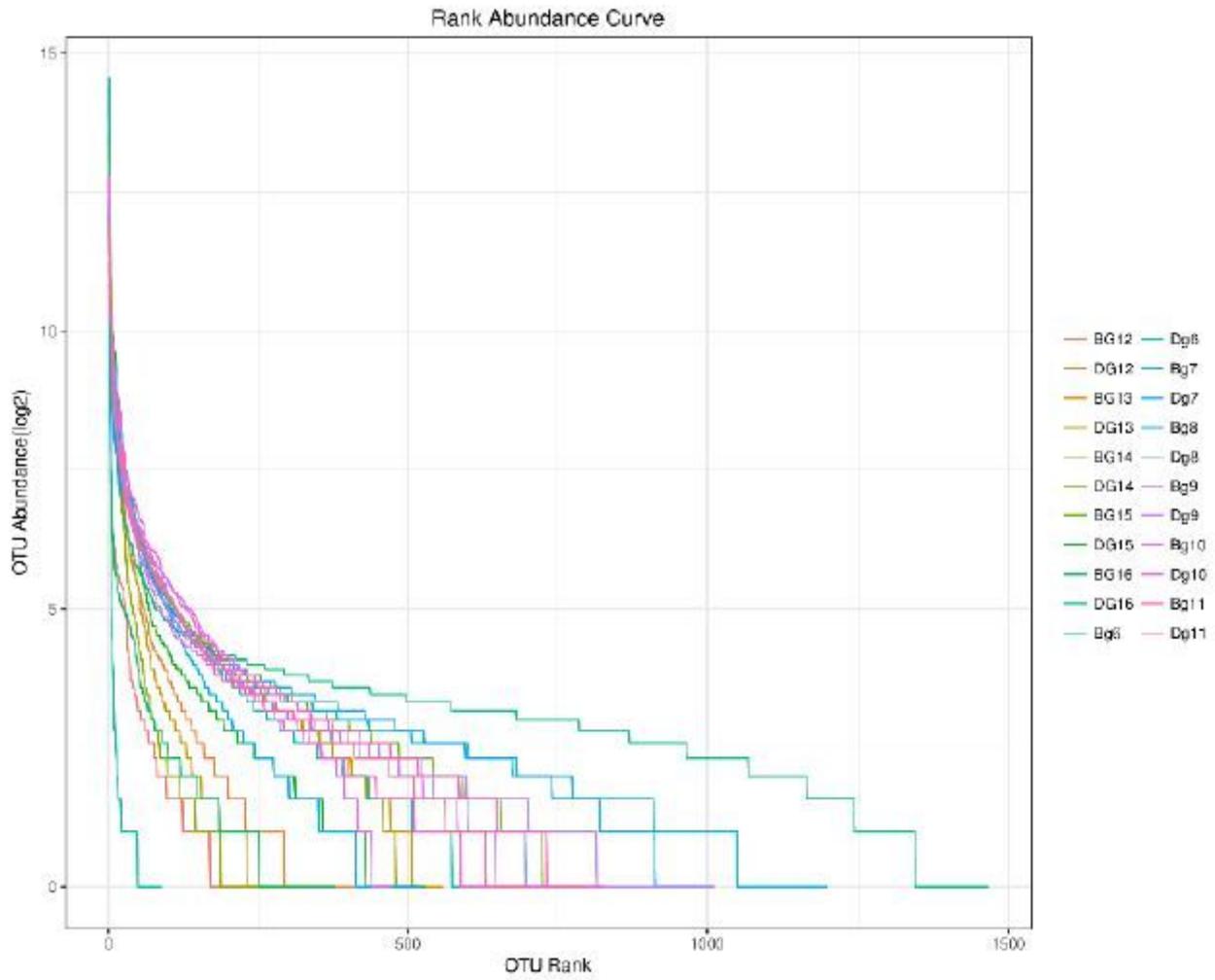


Figure 4

Rank-Abundance curve

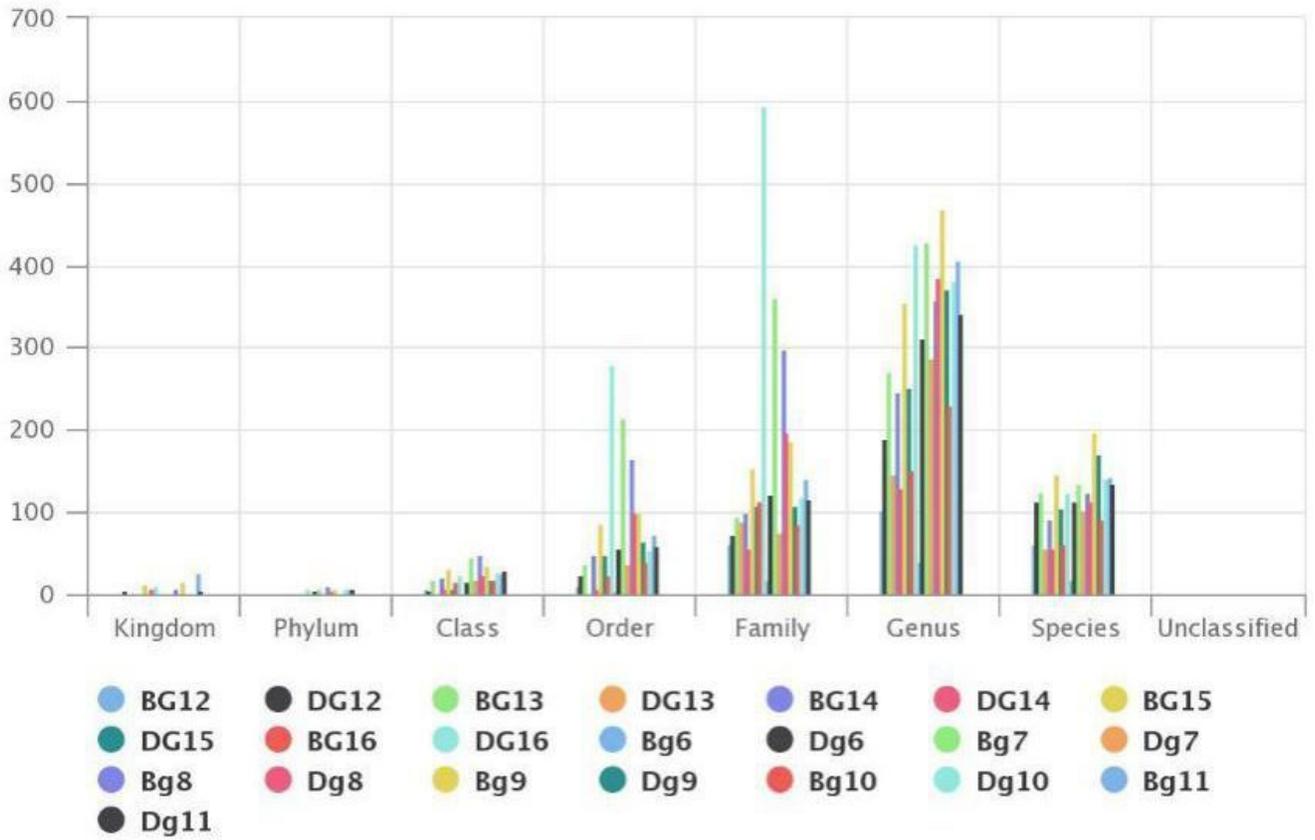


Figure 5

Distribution of sequences per taxonomic level per sample. Statistics of the number of sequences in each taxon level can be used to evaluate the species annotation resolution of each sample. The higher the proportion of annotation to genus and species, the better the annotation effect. Through Figure 6, we can see intuitively that the proportion of each sample at the genus and species level is high, especially at the genus level, and the proportion at the gate level is low, which indicates that the species annotation resolution of annotation results is high, and it also provides a good database for subsequent analysis.

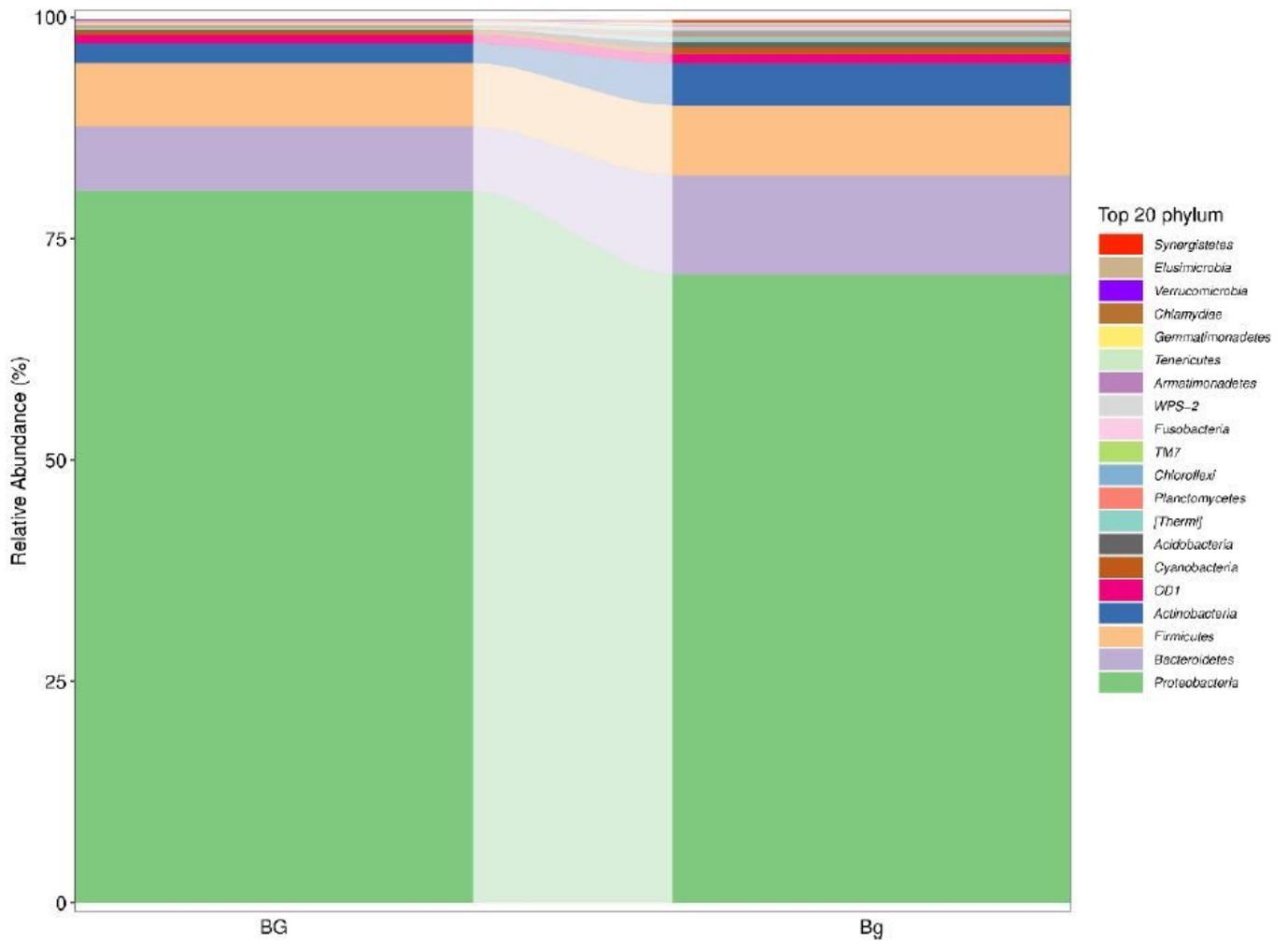


Figure 6

TOP20 species distribution at phylum level of all bile samples

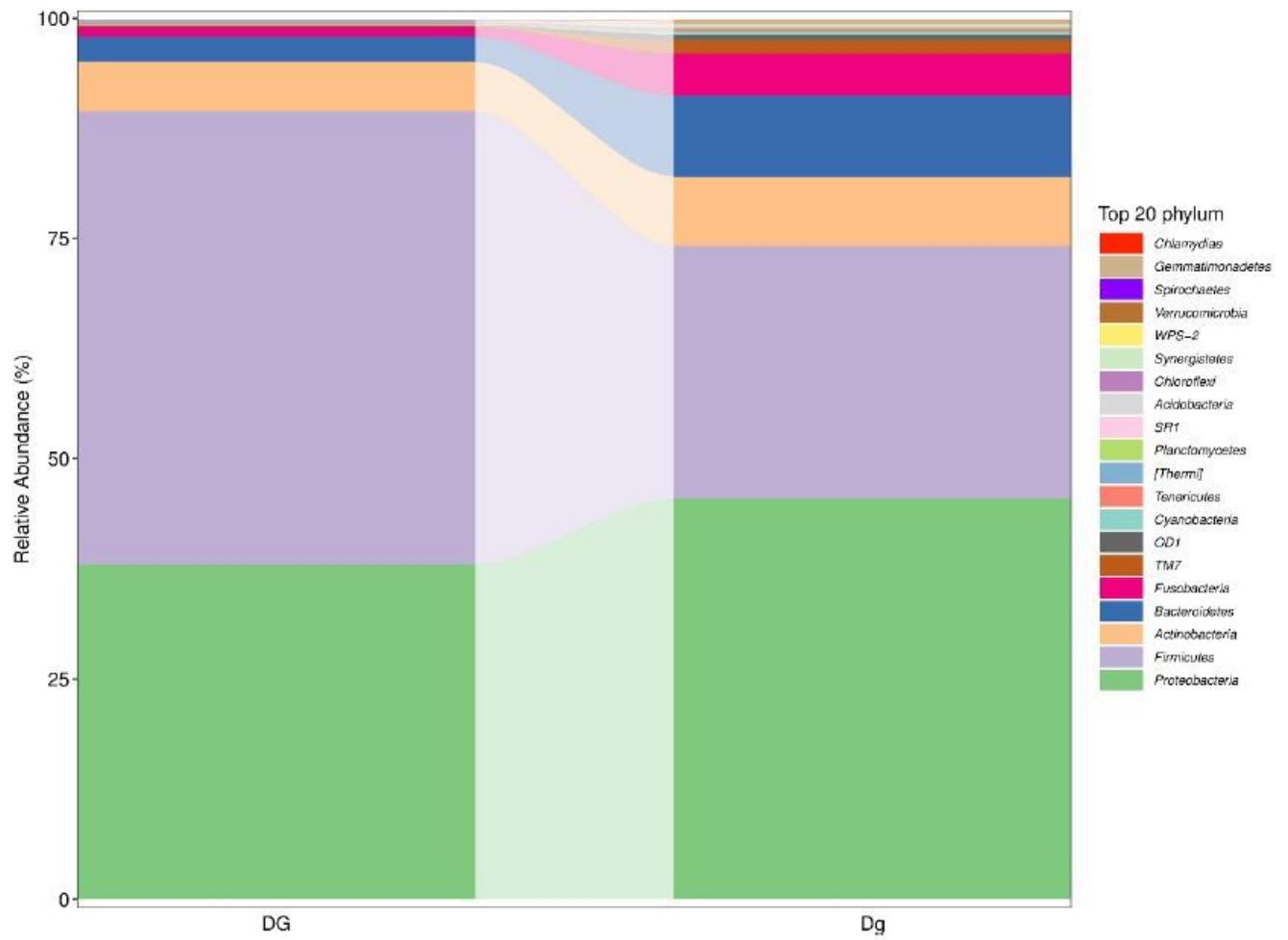


Figure 7

TOP20 species distribution at phylum level of all Intestinal samples

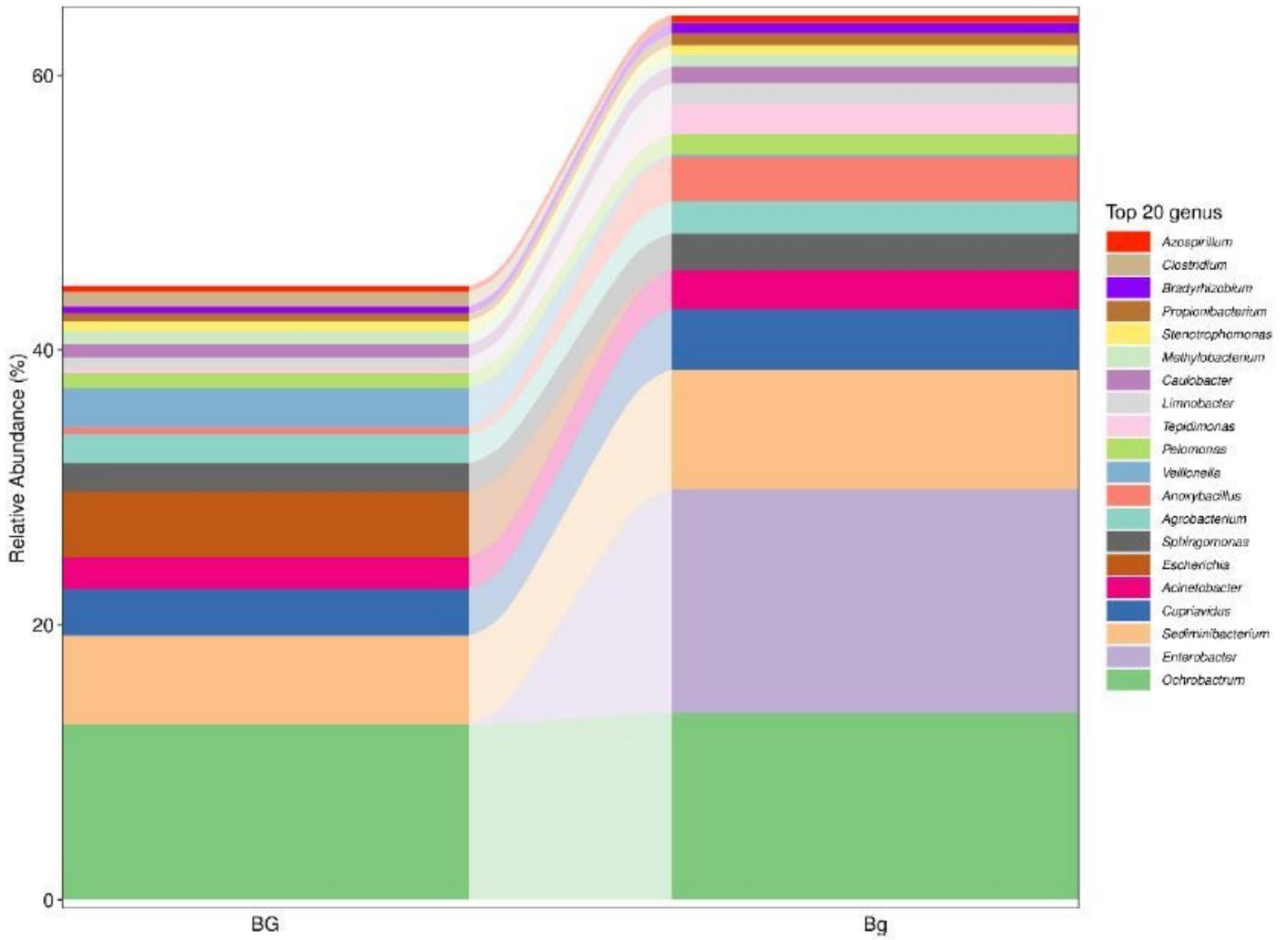


Figure 8

TOP20 species distribution at genus level of all bile samples

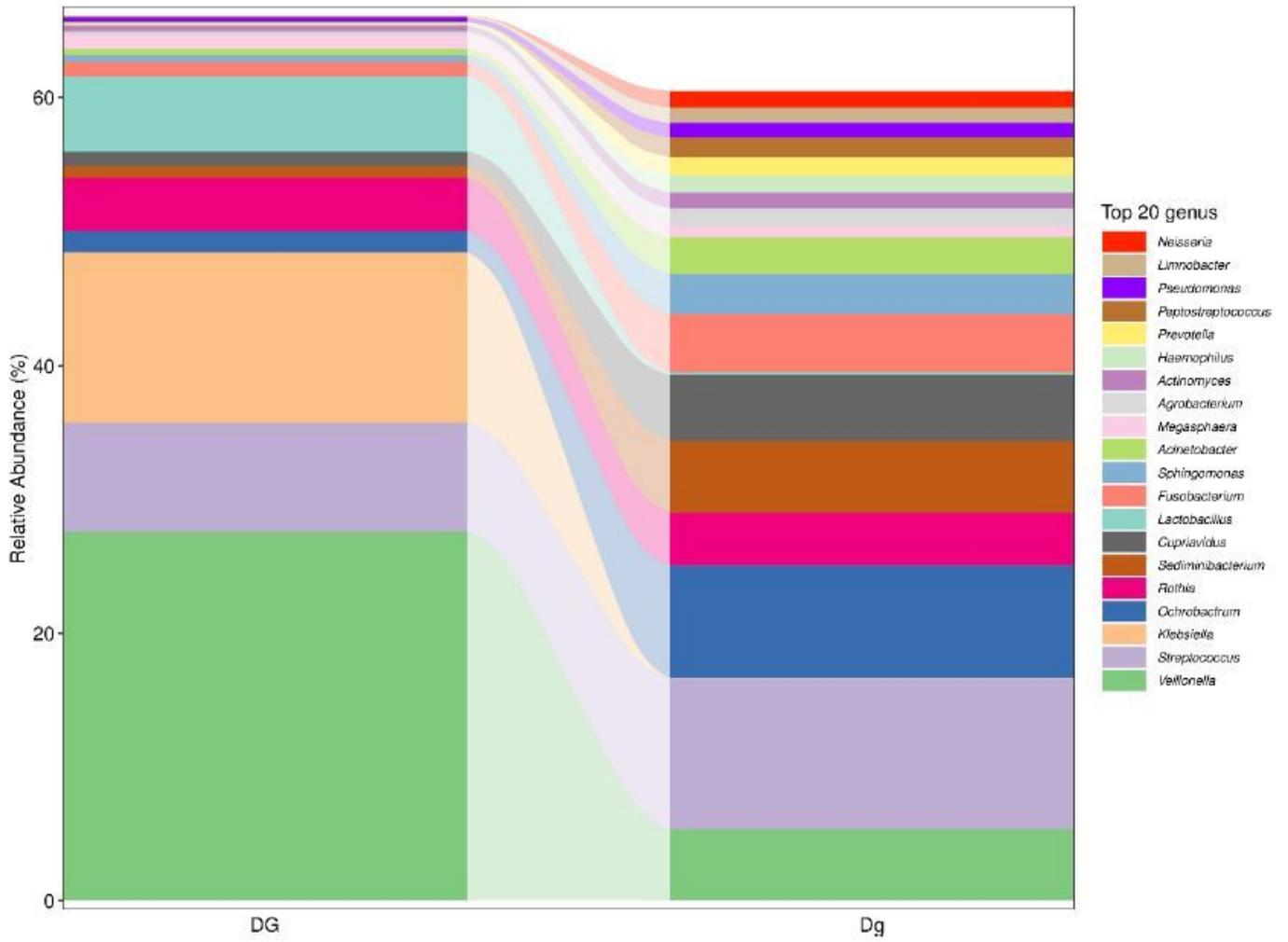


Figure 9

TOP20 species distribution at genus level of all Intestinal samples

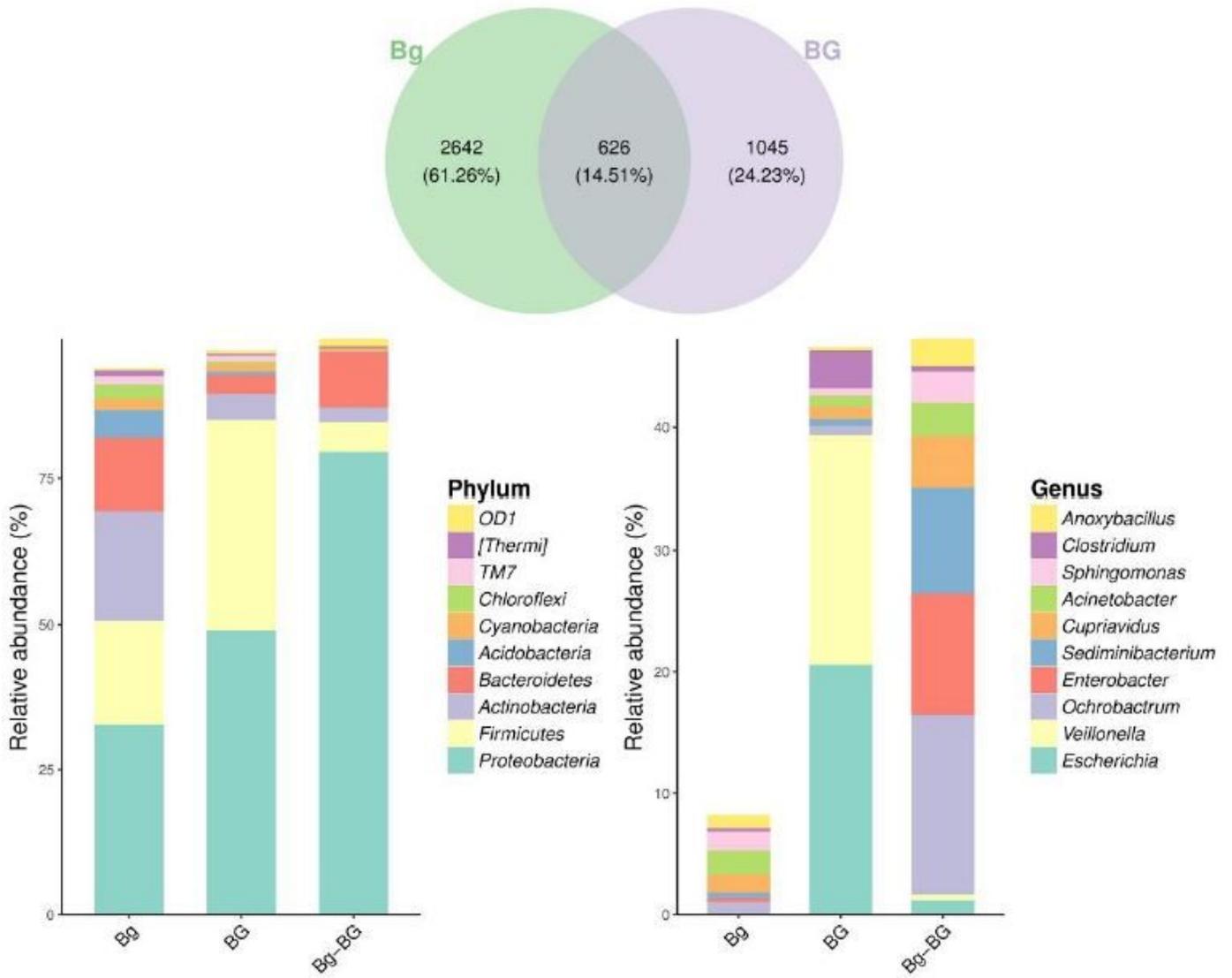


Figure 10

Venn diagram of BG and Bg groups and ASV/OTU abundance

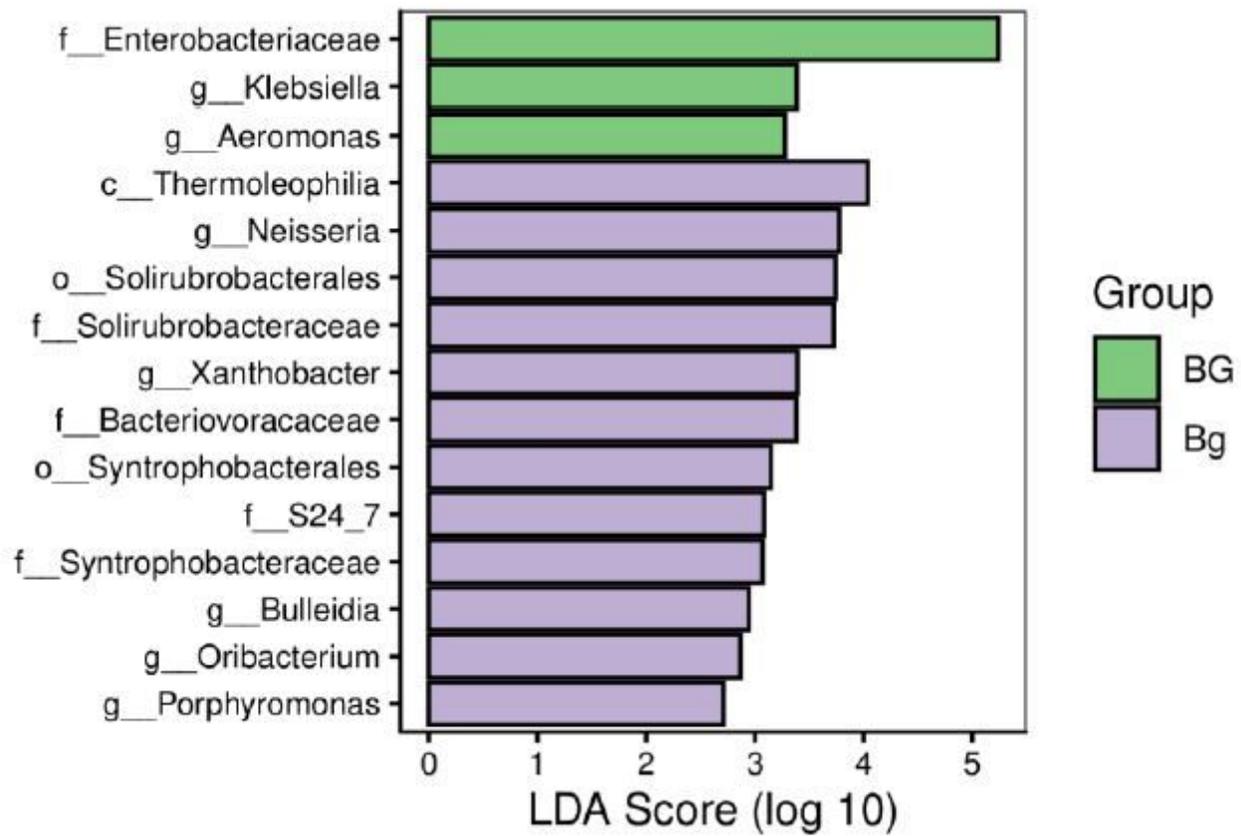


Figure 11

The bar chart of LDA effect value of marker species *Klebsiella*, and *Aeromonas* are unique in the BG Group. histograms of common sequences at phylum and genus level