

Metagenomic Study of the Liver Microbiota in Liver Cancer-Metagenomic and Metatranscriptomic Analyses of the Hepatocellular Carcinoma-Associated Microbial Communities and the Potential Role of Microbial Communities in Liver Cancer

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Abstract

Objective: Metagenomics is a new science that revolutionized microbiology for its ability to study the microbiota of a given environment without the need of culture. Human microbiota is the collection of microbes that inhabit different sites of the human body and recently its alterations were related to different human diseases especially cancers. Liver cancer incidence is continually increasing in Egypt with a high mortality rate. This study aimed to identify the abundant microbial communities that inhabit the liver of the hepatocellular carcinoma patient and may be associated with disease incidence or at least disease progression.

Methods: Fresh liver biopsy samples of two hepatocellular carcinoma Egyptian patients were obtained. DNA from one sample and RNA from other sample were extracted followed by Illumina sequencing. Taxonomic and functional analyses were performed using the MG-RAST server.

Results: Proteobacteria was the dominant phylum followed by Firmicutes and Actinobacteria in both DNA and RNA samples. Some other phyla as Chlorobi, Bacteria and Cyanobacteria had the same abundance in the two dataset. But it was noted that the bacterial diversity and presence of useful bacteria in sample 2 of grade 1 disease (RNA sample) were more than it in sample 1 of grade 2 disease (DNA sample). Also, some other phyla are found in the cDNA dataset annotations. On the other hand, infectious diseases pathways analysis showed the enrichment of infectious diseases pathways of *Staphylococcus aureus* infection, *Vibrio cholera* infection, pathogenic *Escherichia coli* infection, Hepatitis c, Tuberculosis, Epithelial cell signalling in *Helicobacter pylori* infection, Bacterial invasion of epithelial cells, and salmonella infection.

Conclusions: There is a potential link between some definite microbial communities and liver cancer that need some attention for improving early diagnosis and preventing disease progression. Further studies are required to support this.

Keywords: Bacterial translocation; Gut microbiota; Hepatocellular carcinoma; Human microbiome; Liver cancer; Metagenomics; Metatranscriptomics

Introduction and Review of Relevant Literature

All surfaces of the human body, including the skin, mucosal surface and genital and gastrointestinal tracts are occupied by habitat-specific microorganisms whose cells outnumber those of the human host by ten times [1]. The human 'metagenome' is a composite of *Homo sapiens* genes and genes present in the genomes of the trillions of microbes that colonize our adult bodies. 'Our' microbial genomes (the microbiome) encode metabolic capacities that we have not had to evolve wholly on our own [2]. This means that the human genome is only one of the many genomes that affect *Homo sapiens* function. In reality, the organism we call *Homo sapiens* is controlled by a metagenome, a tremendous number of different genomes working in parallel [3]. Recent studies have shown that microbiota imbalances (dysbiosis) are associated with a wide variety of health problems. Inflammatory bowel diseases, obesity, atopic syndromes, various forms of colitis, and even autism, have been linked to disruptions in human-associated microbiota [4]. Metagenomics is an applied science that deals with throughput analysis of Environmental Genomic isolates [5]. It is a rapidly evolving field that emerged from rapid advances in DNA sequencing methods, with a focus on the use of culture-independent methods to study the structures, functions and dynamic operations of microbial communities [6]. Recent

metagenomic assays have shown that human microbial communities can be used as biomarkers for host factors such as lifestyle and disease [7]. The complement of gene transcripts produced by the organisms in a particular environment under particular conditions (the transcriptome of the notional "meta-organism") is the metatranscriptome. As well as representing a "giant microarray experiment" (or more accurately a giant RNA-Seq experiment), metatranscriptomics is also useful for elucidating the presence of highly-expressed genes which might have been missed by chance when sampling the metagenome itself [8]. The use of meta-transcriptomics facilitates detection of novel organisms with pathogenic potential to human and animals [9].

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Liver diseases have become one of the most important causes of morbidity and mortality in the world. The gut microbiota is associated with a number of hepatic diseases from hepatocellular lipid accumulation to stellate cell activation, immune cell recruitment, and cancer development [10]. There is a strong relationship between liver and gut: the portal system receives blood from the gut, and intestinal blood content activates liver functions. The liver in turn, affects intestinal functions through bile secretion into the intestinal lumen [11]. Recent studies have proved that addition of a single agent to the gut microbiome can disrupt enterohepatic homeostasis and promote liver cancer [12].

Hepatocellular carcinoma (HCC) is one of the most common malignant tumors worldwide [13]. HCC in Egypt affects between 5 and 7 cases per 100,000 annually with a mortality rate of 6 per 100,000 reflecting a high disease fatality [14]. Major etiologic factors associated with HCC include infection with hepatitis C (HCV) and hepatitis B (HBV) viruses, excess alcohol intake and aflatoxin B1 exposure [15]. Lehman et al. calculated age- and sex-specific and age- and sex-standardized HCC incidence rates for the eight districts in Gharbiah. They also compared rates from Gharbiah with the USA (US Surveillance Epidemiology and End Results [SEER] database). The findings of this study document the heterogeneous distribution of HCC at regional and international levels [16]. Increasing evidence suggests that derangement of gut flora is of substantial clinical relevance to patients with cirrhosis. Recent studies suggest that, gut flora contributes to the pro-inflammatory state of cirrhosis even in the absence of overt infection. Furthermore, manipulation of gut flora to augment the intestinal content of lactic acid-type bacteria at the expense of other gut flora species with more pathogenic potential may favourably influence liver function in cirrhotic patients [17]. Several recent reports have shown that HCC can occur in patients with longstanding Crohn's disease (CD) in the absence of other underlying high-risk liver diseases [18]. Preclinical and clinical studies over the last decade have suggested a role for bacterial translocation (BT) in the pathogenesis of nonalcoholic steatohepatitis (NASH) [19]. Bacterial translocation (BT) is a phenomenon in which live bacteria or its products cross the intestinal barrier [20] to the mesenteric lymph nodes, systemic circulation, or extraintestinal organs [21]. Non-alcoholic fatty liver disease (NAFLD) comprises a spectrum of diseases ranging from simple steatosis to non-alcoholic steatohepatitis (NASH), fibrosis, and cirrhosis [22]. The liver is continually exposed to gut derived factors including bacteria and bacterial components. To combat this influx, the liver contains a large number of resident immune cells. Together, these immune cell populations in conjunction with other nonparenchymal cells orchestrate a controlled and organized response to these potentially highly inflammatory factors. However, when normal liver physiology is disrupted and inflammatory cells are activated, gut-derived factors likely augment or exacerbate certain liver diseases leading to enhanced tissue damage and propagation of inflammation [10]. Approximately 80% of hepatocellular carcinomas (HCCs) are preceded by chronic liver disease, hepatic fibrosis and cirrhosis. Chronic liver disease is also accompanied by the translocation of intestinal bacteria and pathogen-associated molecular patterns (PAMPs) —molecular motifs only found in microbes, including intestinal bacteria — which induce inflammatory immune responses [23].

In this study, we thought to investigate and characterize the abundant microbial species from liver biopsies of liver cancer patients. This could point out to a potential role of microbial species in liver cancer occurrence or development. This is performed by sequencing of one DNA sample and one RNA sample after performing microbial

enrichment from two liver cancer patients liver biopsies. Then, bioinformatics analysis is conducted to identify the abundant bacterial species as well as enriched functions and infections associated with these sequences. Several bacterial species and enriched infection pathways are identified which could influence the oncogenic transformation of the liver.

Methods

Subjects

Liver biopsy samples were obtained from two donors where Liver biopsy was a part of their requested diagnosis procedures for hepatocellular carcinoma, at the National Cancer Institute in Cairo under supervision of Prof.Dr.Abdel Hady Abdel Wahab and Prof. Dr.Ikram Hamid. The Liver biopsies were obtained under informed consent and the approval of the medical research ethics committees at the National Research Centre and the National Cancer Institute. Liver samples were frozen immediately in liquid nitrogen till used.

Total DNA extraction and purification (Metagenomic study on subject 1)

Total DNA was extracted from biopsy sample of subject 1 using the QIAamp DNA Mini Kit According to the manufacturer protocol followed by ethanol purification.

Total RNA extraction and microbial mRNA enrichment (Metatranscriptomic study on subject 2)

Total RNA was extracted from the second biopsy sample of subject 2 using Thermo Scientific GeneJET RNA Purification Kit According to the manufacturer protocol of RNA purification from mammalian tissues. Ambion® DNA-free™ kit was used to remove contaminating DNA from RNA preparations, and to subsequently remove the DNase and divalent cations from the sample. Microbial RNA enrichment was done using Ambion® MICROBEnrich™ Kit. Because rRNA represents more than 90% of the bacterial RNA, Enrichment of bacterial mRNA from purified total RNA was performed using Ambion® MICROBExpress™ Kit.

DNA and RNA samples preparation and sequencing

Sample preparation and sequencing were done in collaboration with Malaysia Genome Institute

DNA sample preparation was done using Nextera XT DNA sample preparation kit according to user guide and RNA sample preparation was done using the TruSeq® RNA Sample Preparation v2 kit according to Low Sample (LS) protocol

Cluster generation and sequencing

Cluster generation reagents were prepared using TruSeq® PE Cluster Kit v3 and Sequencing was performed on Illumina HiSeq 2000 machine according to user guide.

Bioinformatics analyses

The resulted data files were uploaded to the MG-RAST (Meta Genome Rapid Annotation using Subsystem Technology, v3.3.5.4) server at (<http://metagenomics.anl.gov>). At the manage inbox section, compressed files were unpacked. Also, join paired -ends option was selected to join the paired end reads of each sample to produce longer reads for analysis. The DNA dataset and the cDNA dataset were submitted as eight MG-RAST jobs for the DNA dataset under the accession numbers 4523587.3 (DNA1), 4523588.3 (DNA2), 4523589.3

(DNA3), 4523590.3 (DNA4), 4523591.3 (DNA5), 4523592.3 (DNA6), 4523593.3 (DNA7) and 4523594.3 (DNA8) and as two MG-RAST jobs for the cDNA dataset under the accession numbers 4523596.3 (cDNA1) and 4523597.3 (cDNA2).

Taxonomic analysis

Taxonomic classification of Best similarity hits was reported for the DNA and the cDNA dataset separately by sequence comparison to M5NR protein database (Non-redundant multi-source protein annotation database) and Refseq protein database (NCBI reference sequences database) using a maximum e-value of 1e-5, a minimum identity of 60%, and a minimum alignment length of 15.

Functional analysis

Infectious diseases pathways analysis: The DNA and the cDNA datasets were compared to KO (KEGG orthology) using a maximum e-value of 1e-5, a minimum identity of 60 %, and a minimum alignment length of 15.

Results and Discussion

After demultiplexing, the DNA Dataset was generated as 16 files which is 8 for forward reads (R1) and 8 for reverse reads (R2) (Total size~5 GB), fragment size=350 bp. Whereas, the cDNA dataset was generated as 4 files (2 R1 and 2 R2 reads) (Total size~1.5 GB), and the fragment size=250 bp.

Subject 1 diagnosis was Grade 2 Hepatocellular carcinoma on top of liver cirrhosis and diagnosis of subject 2 was Grade 1 Hepatocellular carcinoma. Bacterial infections are very frequent in advanced cirrhosis and become the first cause of death of these patients [24]. Spontaneous bacterial peritonitis (SBP) is defined as an infection of initially sterile ascitic fluid (AF) without a detectable, surgically treatable source of infection. It is a frequent and severe complication of cirrhotic ascites, first described in the middle of the 1960s [25]. Also, Patients with cirrhosis have altered and impaired immunity, which favours bacterial translocation [26].

In the current study, Analysis results (Figures 1 and 2) showed that bacteria accounted for 16% of DNA sequences that passed the

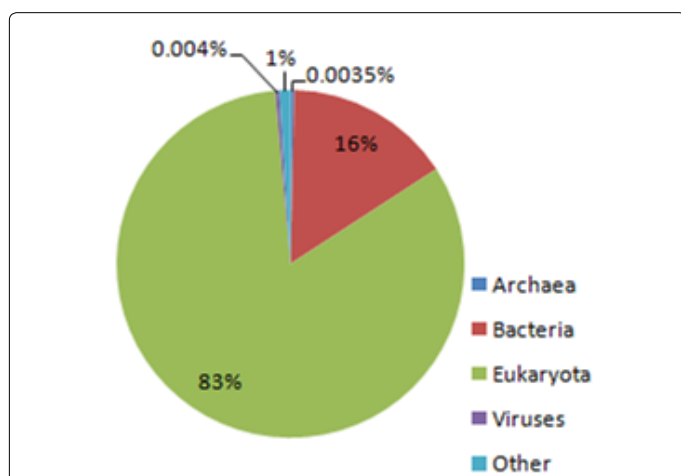


Figure 1: Taxonomic domain distribution of the DNA dataset. The pie chart illustrates the distribution of taxonomic domains for the annotations of the DNA dataset. Each slice indicates the percentage of reads with predicted proteins and ribosomal RNA genes annotated to the indicated taxonomic level. This information is based on all the annotation source databases used by MG-RAST.

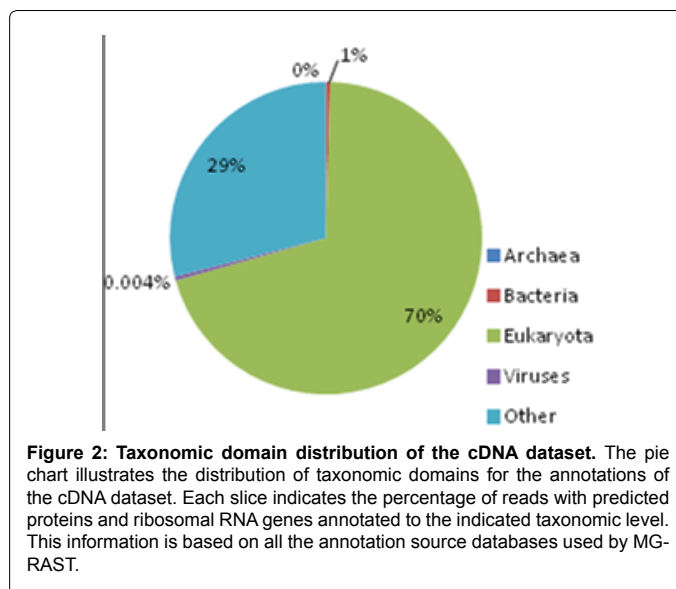


Figure 2: Taxonomic domain distribution of the cDNA dataset. The pie chart illustrates the distribution of taxonomic domains for the annotations of the cDNA dataset. Each slice indicates the percentage of reads with predicted proteins and ribosomal RNA genes annotated to the indicated taxonomic level. This information is based on all the annotation source databases used by MG-RAST.

QC and 1% of cDNA sequences that passed the QC. The noticeable difference between the percentage of bacterial sequences between the DNA and the cDNA sequences probably resulted from the more quick degradation of bacterial mRNA than eukaryotic mRNA [27].

Annotation of taxonomic affiliation of DNA and cDNA sequences with M5NR and Refseq databases of MG-RAST showed that the dominant bacterial phylum in the two samples is Proteobacteria followed by Firmicutes and Actinobacteria as shown in Figures 3-6. Some other phyla as Chlorobi, Bacteria and Cyanobacteria had the same abundance in the two dataset. Also, Bacteroides and some other phyla are found in the cDNA dataset annotations. The Proteobacteria are a major group (phylum) of bacteria. They include a wide variety of pathogens, such as *Escherichia*, *Salmonella*, *Vibrio*, *Helicobacter*, and many other notable genera [28].

According to both M5NR and Refseq annotations in the current study, some specific genera were specified in both DNA and cDNA datasets as shown in the taxonomic Tables 1-4 (Included as supplementary data)[see Additional file.doc]. These genera included: *Chlorobium*, *Acinetobacter*, *Propionibacterium*, *Pseudomonas*, *Micrococcus*, *Nesseria*, *Escherichia*, *Corynebacterium*, *Salmonella*, *Shigella*, *Staphylococcus*, *Enterococcus*, *Lactobacillus*, and *Delftia*.

Klebsiella, *Streptococcus* and *Enterobacter* genera were found in the M5NR and Refseq annotations of the DNA dataset and M5NR annotations of the cDNA dataset. Also *Citrobacter*, *Xanthomonas* and *Aeromonas* were found in M5NR and Refseq annotations of the DNA dataset and Refseq annotations of the cDNA dataset Whereas, *Geobacillus* and *Comamonas* were found in M5NR annotations of the DNA dataset and M5NR and Refseq annotations of the cDNA dataset.

In the same time there was an abundance of *Bacillus*, *Enhydrobacter*, *Paracoccus*, *Burkholderia*, *Serratia*, *Tatumella* and *Mannheimia* in both M5NR and Refseq of the DNA dataset only.

Also, M5NR and Refseq annotations of the cDNA dataset only supported the abundance of *Cyanothece*, *Beggiatoa*, *Brucella*, *Clostridium*, *Treponema* and *Bacteroides* and the presence of *Vibrio*, *Kangiella*, *Legionella* and *Helicobacter* and some other genera.

On the other hand, in the functional analysis, the datasets were annotated with KO of MG-RAST. Cancers, Endocrine and metabolic

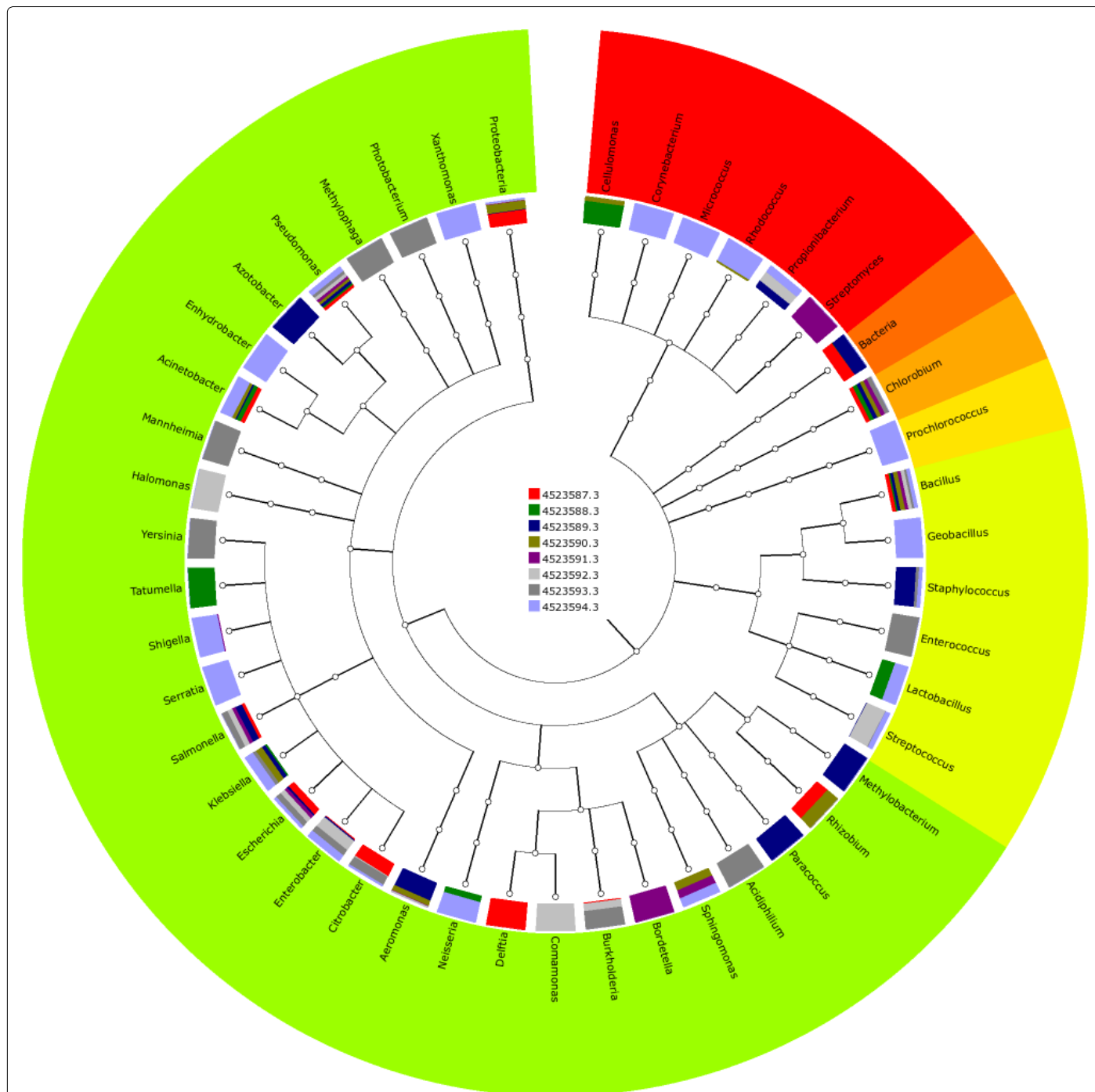


Figure 3: Phylogenetic tree of the bacterial genera detected in the DNA dataset according to M5NR annotations. Figure 3 shows the phylogenetic tree of the bacterial genera detected in the metagenomic study according to M5NR annotations. Genera that belong to the same phylum were colored with the same color. This data was calculated for DNA dataset (4523587.3 (DNA1), 4523588.3 (DNA2), 4523589.3(DNA3), 4523590.3 (DNA4), 4523591.3 (DNA5), 4523592.3 (DNA6), 4523593.3 (DNA7) and 4523594.3 (DNA8)). The data was compared to M5NR using a maximum e-value of 1e-5, a minimum identity of 60%, and a minimum alignment length of 15. Maximum level was chosen to be genus, colored by phylum and the view was restricted to bacteria domain. It is noted from the figure that Proteobacteria is the dominant phylum followed by Firmicutes and Actinobacteria.

diseases, Immune diseases and Infectious diseases had the highest hit numbers in both DNA and cDNA datasets.

Samaras et al. searched Pubmed, Cochrane, and Scopus without time limits for relevant articles to evaluate the evidence regarding the association of such infections with the development of malignancy, excluding the overwhelming evidence of the association of *Helicobacter*

pylori and cancer. They found that there is evidence that some bacterial and parasitic infections are associated with cancer development. The level of evidence of this association varies from high to low; in any case, a long time interval is mandatory for the development of cancer. A high level of evidence exists for the association of *Salmonella typhi* with gallbladder and hepatobiliary carcinoma; *Opisthorchis viverrini*

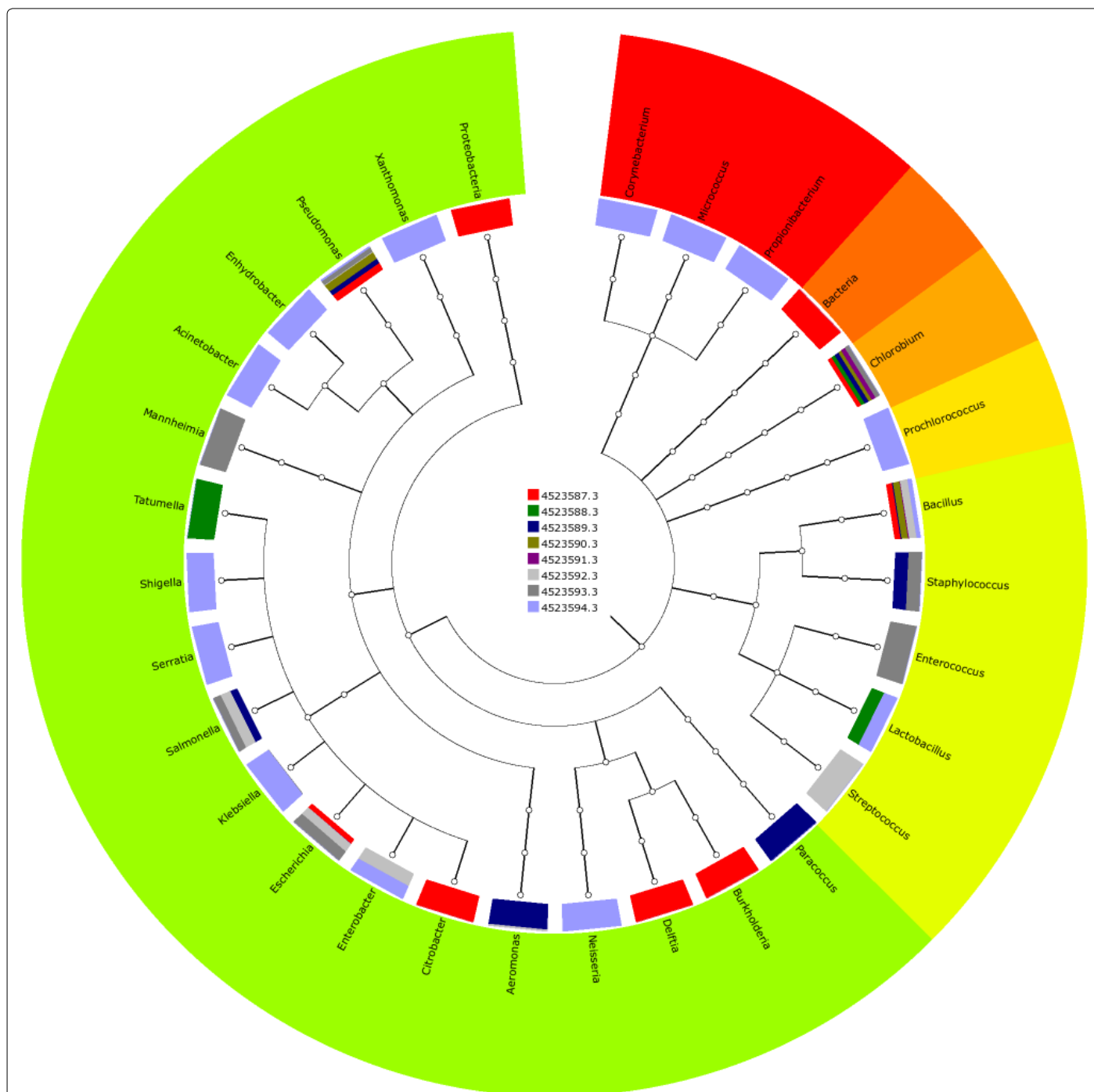


Figure 4: Phylogenetic tree of the bacterial genera detected in the DNA dataset according to Refseq annotations. Figure 4 shows the phylogenetic tree of the bacterial genera detected in the metagenomic study according to Refseq annotations. This data was calculated for DNA dataset (4523587.3 (DNA1), 4523588.3 (DNA2), 4523589.3(DNA3), 4523590.3 (DNA4), 4523591.3 (DNA5), 4523592.3 (DNA6), 4523593.3 (DNA7) and 4523594.3 (DNA8)). The data was compared to Refseq using a maximum e-value of 1e-5, a minimum identity of 60%, and a minimum alignment length of 15. Maximum level was chosen to be genus, colored by phylum and the view was restricted to bacteria domain. The Refseq annotations supported the M5NR annotations and that Proteobacteria is the dominant phylum followed by Firmicutes and Actinobacteria.

and *Clonorchis sinensis* with cholangiocarcinoma and *Schistosoma hematobium* with bladder cancer [29].

Here, in infectious diseases pathways analysis, level 3 KO annotations showed that among the determined infections pathways enriched in both DNA and cDNA datasets were *Staphylococcus aureus* infection, *Vibrio cholera* infection, pathogenic *Escherichia coli* infection, Pertussis,

Hepatitis c and Tuberculosis with a probability value equal 0.0000 for all. Also, African trypanosomiasis (p value=0.0028), Malaria (p value=0.0051), Epithelial cell signaling in *Helicobacter pylori* infection (p value=0.0585), Bacterial invasion of epithelial cells (p value=0.0955), and salmonella infection (p value=0.5000) were detected as shown in Figures 7 and 8. Tables 5 and 6 (Included as supplementary data)

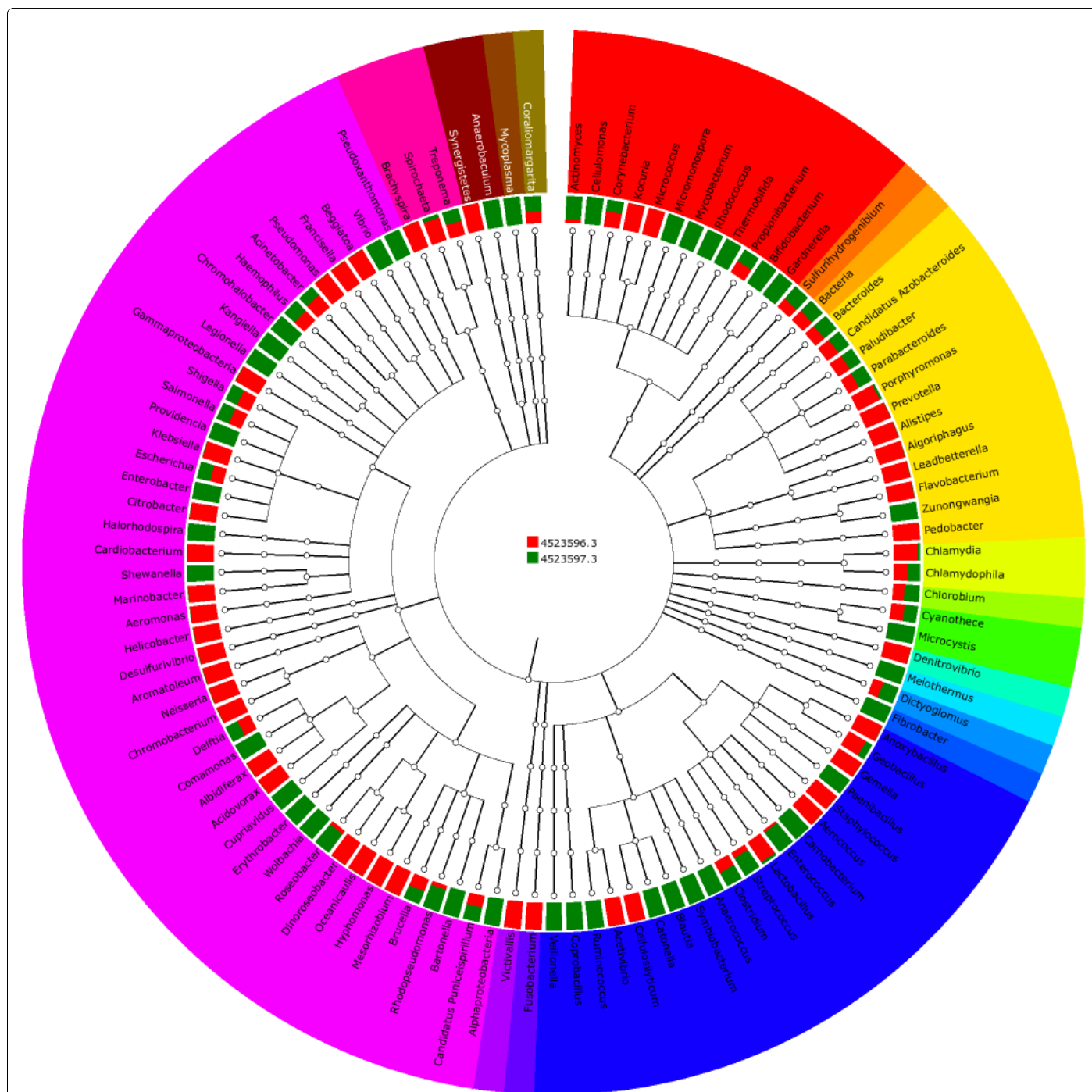


Figure 5: Phylogenetic tree of the bacterial genera detected in the cDNA dataset according to M5NR annotations. Figure 5 shows the phylogenetic tree of the bacterial genera detected in the metatranscriptomic study according to M5NR annotations. This data was calculated for cDNA dataset (4523596.3 (cDNA1) and 4523597.3 (cDNA2)). The data was compared to M5NR using a maximum e-value of 1e-5, a minimum identity of 60%, and a minimum alignment length of 15. Maximum level was chosen to be genus, colored by phylum and the view was restricted to bacteria domain. As shown the phylum Proteobacteria is the dominant followed by Firmicutes and Actinobacteria as in the metagenomic study. Also Bacteroidetes and some other phyla are shown here.

show this in more detail [see Additional file.doc]. Also, Toxoplasmosis, Legionellosis, Shigellosis and Chagas disease were detected in DNA dataset whereas; Amoebiasis and Hepatitis B were detected in cDNA dataset. Infectious diseases pathways analysis showed the expression of anti-inflammation proteins against inflammation caused by some infectious organisms which reveal the potential role of these organisms in disease incidence or by sure in disease progression. Despite

of abundance of *Shigella* and *Legionella* species in the taxonomic classification of cDNA dataset, Shigellosis and Legionellosis were not determined as infectious diseases in functional analysis although, they were determined as infectious diseases in DNA dataset of sample 1 of grade 2 disease. This probably means that those species may lead to disease progression with time. These infections may be also associated with cirrhosis. Bacterial infection is a frequent and severe complication

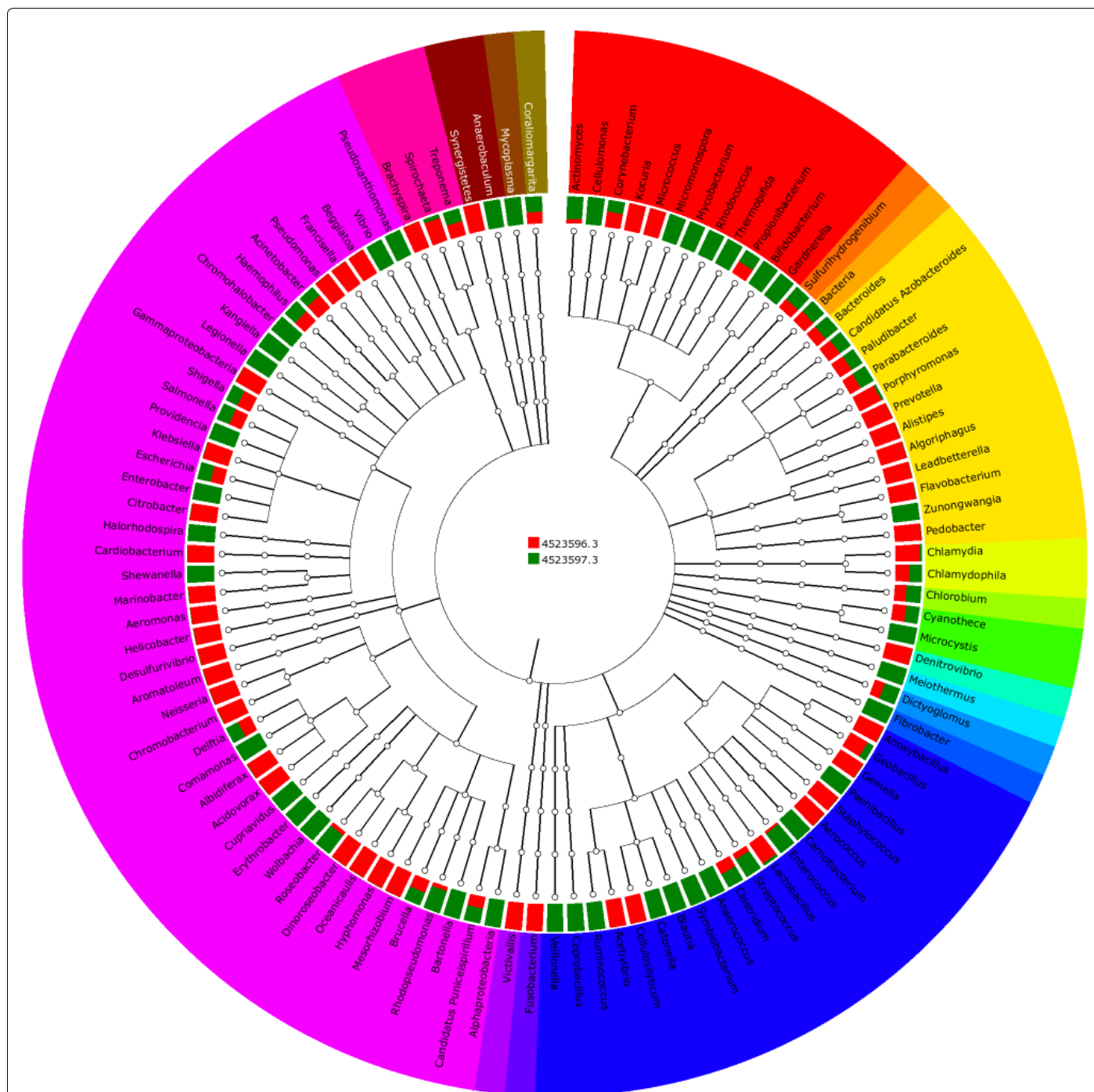


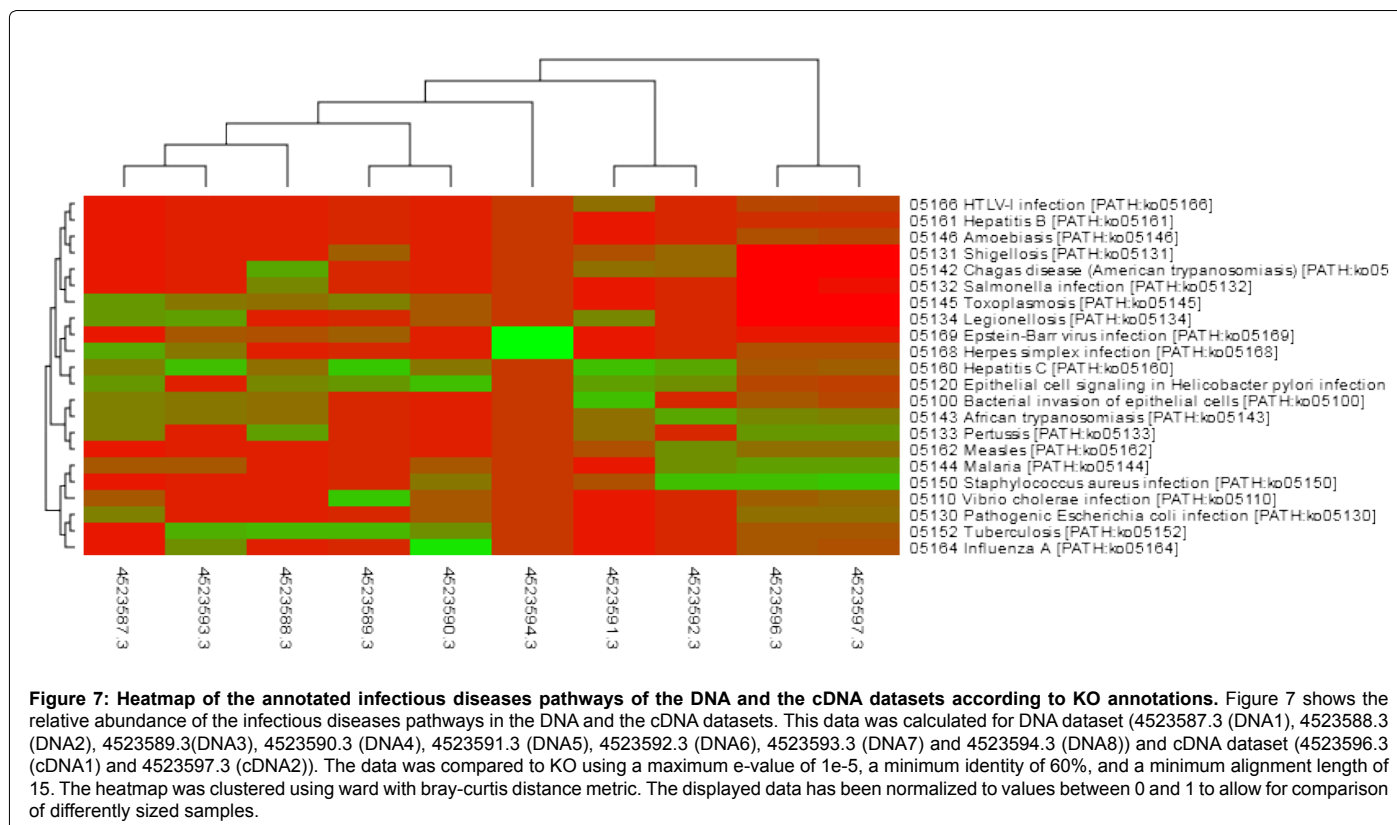
Figure 6: Phylogenetic tree of the bacterial genera detected in the cDNA dataset according to Refseq annotations. Figure 6 shows the phylogenetic tree of the bacterial genera detected in the metatranscriptomic study according to Refseq annotations. This data was calculated for cDNA dataset (4523596.3 (cDNA1) and 4523597.3 (cDNA2)). The data was compared to Refseq using a maximum e-value of 1e-5, a minimum identity of 60%, and a minimum alignment length of 15. Maximum level was chosen to be genus, colored by phylum and the view was restricted to bacteria domain. As shown the Refseq annotations supported the M5NR annotations.

of cirrhosis [30]. Also it was noted that sample one have more aggressive bacterial species from the same genus than sample two and this confirm its correlation with disease development.

However, the more abundance of *Lactobacillus* species in the cDNA dataset (generated from sample 2 of Grade 1 disease) than in the DNA dataset and the abundance of *Bifidobacterium* species in the cDNA dataset may be associated with the positive role of some normal

intestinal microbiota in playing a defensive role against pathogens.

This was in agreement with the results obtained by Singh et al. that investigated the status of bacterial colonization and inflammation in the normal, cirrhotic primary biliary cirrhosis (PBC), and nonalcoholic steatohepatitis (NASH) human liver tissues. Comparatively normal livers showed increased bacterial colonization than PBC and NASH.



They analyzed mRNA levels of Toll-like receptors (TLR) 2 and TLR4, and protein levels of TLR4. Phosphorylated IKK α (pIKK α) protein estimation served as a marker for nuclear factor-kappa B (NF- κ B) activation. In spite of the increased bacterial colonization in normal liver tissues, lower levels of TLR2/4 mRNA and TLR4 and pIKK α proteins were found compared to PBC and NASH indicating the maintenance of suppressed inflammation and immune tolerance in normal livers. They mentioned that this is the first clinical evidence showing suppressed inflammation despite bacterial colonization in normal human livers thus maintaining liver immune homeostasis [31].

The results of the current study supported the analysis results of fecal microbiome in patients with hepatitis B and alcoholic liver cirrhosis that demonstrated an increase in pathogenic *Enterobacteriaceae* and *Streptococcaceae*, while beneficial *Bifidobacteria* and *Lachnospiraceae* were decreased. In addition, liver cirrhosis induced by carbon tetrachloride in rats was also associated with high levels of *Enterobacteraceae*. Another study reported a decrease in Gram-positive anaerobic *Clostridium* groups and an increase in the aerobic/anaerobic bacterial ratio in mice with fibrosis. For animals with cirrhosis, treatments with antibiotics (e.g. norfloxacin) or probiotics (e.g. *Bifidobacterium*) reduces *Enterobacter*, while it increases *Bifidobacterium* and *Lactobacillus*, resulting in decreased systemic endotoxin levels and improved liver function [32]. Yilmaz et al. presented an immunocompetent case of liver abscess caused by *Streptococcus anginosus* originated most probably from oral flora [33]. Spencer et al. performed a 2-month inpatient study of 15 female subjects who were placed on well-controlled diets in which choline levels were manipulated. Variations between subjects in levels of *Gammaproteobacteria* and *Erysipelotrichi* were directly associated with changes in liver fat in each subject during choline depletion. The *Gammaproteobacteria* genera identified in their study, including

Klebsiella spp, *Enterobacter* spp, and *Escherichia* spp, are known gram-negative bacteria with lipopolysaccharide- containing membranes [34].

Although most infectious microorganisms that reach the liver via the blood are rapidly cleared, it is evident from the success of certain pathogens — such as the hepatitis B and hepatitis C viruses and malaria-causing *Plasmodium* spp. in establishing hepatic infections that the liver provides a favourable environment for escaping immune responses [35]. Also, Dapito et al. tested the hypothesis that the intestinal microbiota and Toll-like receptors (TLRs) promote hepatocellular carcinoma (HCC), a long-term consequence of chronic liver injury, inflammation, and fibrosis. Hepatocarcinogenesis in chronically injured livers depended on the intestinal microbiota and TLR4 activation in non-bone-marrow-derived resident liver cells. TLR4 and the intestinal microbiota were not required for HCC initiation but for HCC promotion, mediating increased proliferation, expression of the hepatomitogen epiregulin, and prevention of apoptosis. Gut sterilization restricted to late stages of hepatocarcinogenesis reduced HCC, suggesting that the intestinal microbiota and TLR4 represent therapeutic targets for HCC prevention in advanced liver disease [36]. DeLeon et al. examined whether gallium maltolate (GaM), a novel formulation of Ga, had antibacterial activity in a thermally injured acute infection mouse model. Dose-response studies indicated that a GaM dose as low as 25 mg/kg of body weight delivered subcutaneously was sufficient to provide 100% survival in a lethal *P. aeruginosa*-infected thermally injured mouse model. Mice treated with 100 mg/kg GaM had undetectable levels of *Pseudomonas aeruginosa* in their wounds, livers, and spleens, while the wounds of untreated mice were colonized with over 108 *P. aeruginosa* CFU/g of tissue and their livers and spleens were colonized with over 105 *P. aeruginosa* CFU/g of tissue. GaM also significantly reduced the colonization of *Staphylococcus aureus* and *Acinetobacter baumannii* in the wounds of thermally injured mice. Furthermore, GaM was also

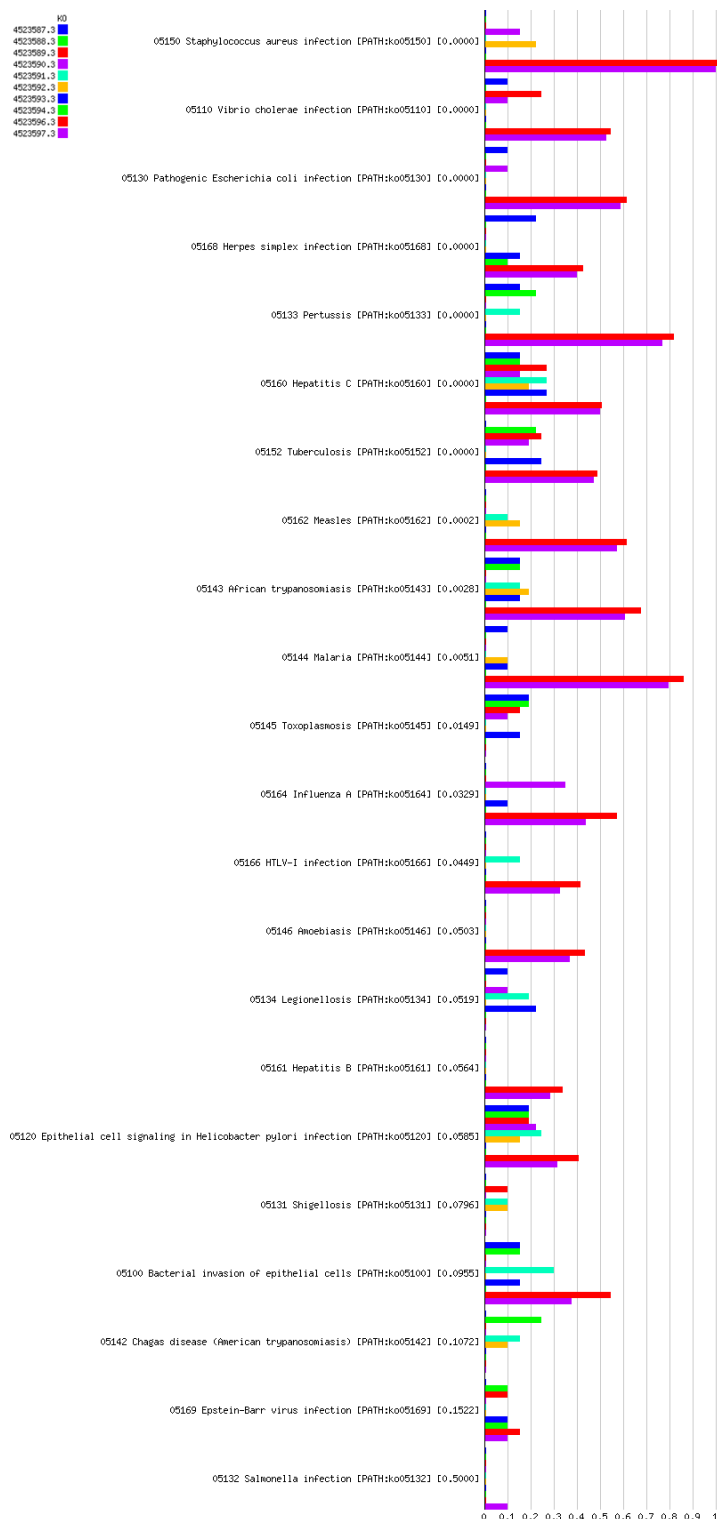


Figure 8: Functional barchart of infectious diseases pathways KO annotations of the DNA and the cDNA datasets. The Functional barchart shows the infectious diseases pathways detected in the DNA and the cDNA datasets according to KO annotations and the probability values of each annotated pathway. This data was calculated for DNA dataset (4523587.3 (DNA1), 4523588.3 (DNA2), 4523589.3(DNA3), 4523590.3 (DNA4), 4523591.3 (DNA5), 4523592.3 (DNA6), 4523593.3 (DNA7) and 4523594.3 (DNA8)) and cDNA dataset (4523596.3 (cDNA1) and 4523597.3 (cDNA2)). The data was compared to KO using a maximum e-value of 1e-5, a minimum identity of 60%, and a minimum alignment length of 15. The data has been normalized to values between 0 and 1. The p-values were calculated using t-test-un-paired and the following groups:

4523587.3 (DNA1) group1; 4523588.3 (DNA2) group1; 4523589.3 (DNA3) group1; 4523590.3 (DNA4) group1; 4523591.3 (DNA5) group1; 4523592.3 (DNA6) group1; 4523593.3 (DNA7) group1; 4523594.3 (DNA8) group1; 4523596.3 (cDNA1) group2; 4523597.3 (cDNA2) group2

therapeutically effective in preventing preestablished *P. aeruginosa* infections at the site of the injury from spreading systemically [37,38].

Conclusions

It can be concluded that there is a potential link between the microbial communities and liver cancer that needs further verification and studies. We conclude also that certain bacterial phyla such as proteobacteria were the most abundant type of bacteria in both samples. This is in agreement with previous studies highlighting the role of *H. pylori* in liver cancer and stomach and gut cancers and pathogenic *E. coli* in gut diseases.

Also, the present study indicates to a contributing probability of some infections in pathogenesis and progression of liver cancer. Also, these data indicate to the key role of bacterial translocation of some intestinal microflora or its products in disruption of liver homeostasis.

This study is a preliminary study with our available resources in order to shed a light on the question of the relation between the gut microbiota and liver cancer. More studies to confirm the conclusions of the paper are needed in the future.

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