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Microbial communities associated with stunted growth syndrome in *Penaeus vannamei* farming

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Abstract

Stunted growth syndrome (SGS) is an emerging concern in *Penaeus vannamei* farming in India and globally, with limited information available on the aetiology, despite its significant economic impact on shrimp farming. Among the various contributing factors, microbial communities in the culture environment play an important role. Hence the present study aimed to profile the microbial communities in pond water from both SGS affected and healthy shrimp ponds. Microbial profiling and their functional prediction were carried out using metagenomic shotgun sequencing. Taxonomic classification was performed with *Kaiju*, while metagenome assembly and functional prediction were conducted using *OmicsBox*. The taxonomic analysis revealed higher dominance of *Oceanospirillum* and *Vibrio* species in SGS-affected pond water, whereas *Rhodobacteraceae bacterium* and *Neptunomonas* species were more abundant in healthy pond water. Alpha diversity analysis indicated a greater diversity in the healthy pond water sample, compared to SGS-affected pond water. Distinct indicator taxa were identified in both healthy and diseased samples each showing considerable abundance in their respective conditions. Functional prediction revealed that genes related to amino acid transport and metabolism, cell wall/membrane/envelop biogenesis, as well as energy production and conversion were predominant. The observed differences in microbial diversity, along with the presence of indicator taxa in healthy and diseased samples, provide insights into the potential role of microbial communities in the manifestation stunted growth syndrome.

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Introduction

The Pacific white shrimp farming across the world is facing several impediments, mainly due to bacterial and viral diseases. Economic losses due to the diseases in India accounted for an annual loss of US$ 1.02 B (Patil *et al.,* 2021). Stunted growth syndrome (SGS) is among one of the most commonly observed diseases in *P. vannamei* farming. SGS, a phenomenon of sub-optimal growth observed in shrimps, was first reported in *P. vannamei* in 1989 and subsequently in *Penaeus monodon* during early 2000's (Kooloth *et al.,* 2021). This condition does not cause mortality but the retarded growth of the animals contributes to severe economic loss. Genetics, environment, and many infectious agents especially the microsporidian *Enterocytozoon hepatopenaei* (EHP) were reported to play a role in the manifestation of this disease (Rajendran *et al.,* 2016; Kooloth *et al.,* 2021). However, SGS can even manifest from the complex interplay between the host, microbes and environment even in the absence of known infectious agents.

Shrimp health is significantly impacted by their environment *i.e.,* the pond water, and the microbes present in them. Pond water microbiota plays an important role in nutrient cycling, maintenance of water quality and health of the farm-reared shrimp (Huang *et al*., 2018). Significant correlations were reported between rearing-water bacterial communities and the intestinal microbiota of shrimps in a biofloc-based culture system (Cardona *et al*., 2016). There has been increasing evidence on the role of gut microbiota on important functions

like immunity, health regulation and nutrient absorption in shrimps (Li *et al*., 2018, Fan *et al.,* 2019, Liu *et al.,* 2019, Servin *et al.,* 2021). Hence, understanding microbial communities present in the culture environment is important in case-control studies to derive causal relations on the manifestation of diseases. Conventional methods of isolation, culture and imaging techniques cannot be employed for complete profiling of environmental microbiota as majority of the microorganisms, around 99%, are unculturable. To bridge this gap, metagenomics is employed to sequence all the microbiota including the unculturable ones (Forbes *et al.,* 2017; Quince *et al.,* 2017).

Metagenomics applications in aquaculture include microbial profiling, diversity analysis, identification of antibiotic resistance genes (ARGs), novel and potential pathogens, microbial associations with bioflocs and probiotics (Martínez *et al.,* 2017). Studies on microbial associations with shrimp diseases were conducted in recent years on white faeces syndrome (Hou *et al.,* 2018), intestinal disorders (Xiong *et al.,* 2015) and acute hepatopancreatic necrosis disease (AHPND) (Cornejo *et al.,* 2017, Hossain *et al.,* 2021). However, there are no microbial association studies conducted on SGS and this study is the first attempt in this direction.

This study is aimed at exploring the possible relationship between microbiota present in rearing water and SGS in *P. vannamei* through shotgun metagenomics. Comparative assessment of microbial communities of pond water form both SGS affected and healthy shrimp ponds was conducted to understand their presence and functions. Microbial signatures of SGS presented in this study are intended to provide a deeper understanding of the manifestation of this yield-limiting syndrome.

Materials and methods

Sample collection

Water samples from *Penaeus vannamei* culture ponds, both affected and unaffected by SGS, were collected from the farms located at Nellore (14.44 N; 79.98 E), in Andhra Pradesh, India. At the time of collection, ponds were at 70 days of culture and salinity levels were around 23 ppt. Both water and animal samples were collected from the pond experiencing SGS (disease) and normal ponds (healthy). One pond sample representing healthy and SGS infected were retained for metagenomic sequencing, discarding all the disease samples with known causative agents. In the selected ponds, animal samples were tested negative for known pathogens like white spot syndrome virus (WSSV), infectious hypodermal and hematopoietic necrosis (IHHNV) and *Enterocytozoon hepatopenaei* (EHP). Size variations observed in the SGS infected ponds is shown in Fig. 1.

Sample preparation, DNA isolation and sequencing

Water samples of 10 l stored at 4°C were used for microbial DNA isolation. Five litres of water from infected and healthy ponds were centrifuged at 5000 *g* for 15 min at 4°C as per the protocol followed by Kumar *et al.* (2013). The supernatant was collected in a sterile container. Pellet was suspended in 50 ml sterile seawater and stored at 4°C until further use. Supernatant was filtered through a 0.22 µ filter paper by vacuum filtration to collect suspended microbes. The

Fig. 1: Shrimps affected with stunted growth syndrome from the same pond

filtrate was scrapped and collected in a sterile 2 ml microcentrifuge tube. DNA was isolated from 0.25 g of microbial samples using the PowerSoil® DNA Isolation Kit (Qiagen). Sequencing libraries were prepared using the NEBnext ultra DNA library preparation kit. The quality of the library was checked using an Agilent TapeStation. Libraries were sequenced using the Illumina HiSeq2500 platform and Paired-end reads with 250 base pairs length were generated for both the samples.

Taxonomic classification and alpha diversity analysis

Raw reads obtained from the sequencer were subjected to different shotgun metagenomic data processing methods like quality assessment and control, taxonomic classification, assembly, differential abundance analysis, functional annotation, diversity analysis, virulence, and resistant gene predictions. Quality testing tool FastQC v0.11.8 (Andrews *et al.,* 2010) was used to obtain an overview of the quality metrics of the raw reads. These reads were trimmed for low quality and adapter content using Trimmomatic v0.39 (Bolger *et al.,* 2014) and retained sequences having a minimum length of 50 bases. Then the high quality reads were subjected to taxonomic classification using the standalone version of Kaiju v. 1.7.3 (Menzel *et al.,* 2016). Kaiju was run with default parameters against the latest prokaryotic non-redundant protein database along with fungi and microbial eukaryotes (nr+euk). Microbial diversity analysis was carried out using R library 'vegan' (Oksanen *et al.,* 2013) with a threshold of 0.1% minimum abundance to estimate Shannon, Simpson, Inverse Simpson and fisher alpha indices.

Metagenome assembly and annotation

Further, high quality reads were subjected to assembly and annotation using metagenomics pipeline in OmicsBox (BioBam, 2019) software. Omicsbox implementation of metaSpades v.3.15.2 (Nurk *et al.,* 2017) for metagenome assembly, Prodigal (Hyatt *et al.,* 2010) for prediction of open reading frames (ORFs), and, EggNOG (Jensen *et al.,* 2008) mapping for annotation of orthologous groups of genes were used in this study. Predicted ORFs were searched against the virulence factor database (VFDB) (Chen *et al.,* 2005) to identify candidate virulent genes with an e-value set at 1e-6. Venn diagram was plotted using online tool http://bioinformatics.psb.ugent.be/webtools/Venn/.

Data availability

The metagenomic sequencing data of this study are deposited in the SRA repository of Genbank with the accession number PRJNA686191.

Results

Sequence statistics

Shotgun sequencing of the samples generated 8,963,000 and 8,445,407 raw sequence reads for both healthy and disease samples respectively. The quality trimming filtered around 30% of the reads from both the samples resulting in high quality reads for further processing (Table 1). Around 70% of the reads have been retained post QC with minimum and maximum read lengths of 50 and 250 bp respectively. GC content of the healthy and diseased samples was found to be 54 and 45% respectively.

Taxonomy and diversity indicators

The taxonomic classifications were assigned by Kaiju for 65 and 74% reads out of the total reads subjected to analysis for healthy and disease samples respectively. *Proteobacteria, Bacteroidetes*, *Actinobacteria*, *Firmicutes* and *Chlamydiae* were found to be the most dominant phyla across samples. The classified reads of the healthy sample comprised 76.4% *Proteobacteria*, 11.6% *Bacteroidetes* and 1.8% *Actinobacteria*, whereas the diseased sample comprised 55.20% *Proteobacteria*, 31.32% *Bacteroidetes*, 3.9% *Actinobacteria*, 2.44% *Firmicutes* and 2.03% *Chlamydiae* as the dominant/major phyla (Fig. 2a). Family level abundances between healthy and diseased samples depicted in Fig. 2b clearly indicated higher abundances of *Oceanospirillaceae*, *Flavobacteriaceae*, *Crocinitomicaceae* and *Vibrionaceae* in diseased sample in comparison to healthy sample. *Rhodobacteraceae* and *Pseudoalteromonadaceae* exhibited their prominence in healthy samples.

Table 1. Sequence statistics

	Healthy	Diseased
Raw reads	8963000	8445407
Clean reads	6291889	5856013
Sequence length (bp)	250	250
Sequence length of clean reads (bp)	50-250	50-250
GC (%)	54	45

Rhodobacteraceae bacterium (Unclassified), *Neptunomonas*, *Vibrio*, *Idiomarina* and *Marivita*, were among the major genera associated with healthy samples which constituted 19.3% of total genera. Whereas, *Oceanospirillum*, *Vibrio*, *Fluviicola*, *Salegentibacter* constituted 34.1% of total genera present in diseased samples. The diseased sample had a higher percentage of *Vibrios* (5.9%) than the healthy sample (4.9%) (Fig. 2c). Species-level abundances revealed that the healthy sample was dominated by *Rhodobacteraceae bacterium EhC02* (15.7%), *Neptunomonas concharum* (2.1%), *Idiomarina atlantica* (1.5%), whereas the diseased sample was dominated by *Oceanospirillum sactuarii* (15.5%), *Vibrio aphrogenes* (4.2%), *Fluvicola* sp. (4.2%), *Salegentibacter mishustinae* (1.8%) and *Oceanospirillum maris* (1.7%) (Fig. 2d). A total of 25,028 taxa were found to be common to both the samples, out of which 6193 taxa specific to healthy sample and 2106 specific to diseased sample (Fig. 3).

The diversity indices to assess the species richness within and between samples were calculated. As shown in Table 2 the estimates for Shannon index, Simpson index and fisher alpha were slightly higher for healthy samples compared to diseased samples, which indicates richer diversity in the healthy sample than that of the diseased sample.

Metagenome assembly and annotation

Metagenome assembly carried out with metaSPAdes assembler revealed a total of 16,315 and 8,503 contigs with an N50 value of 5,823 and 12,482 for healthy and diseased groups respectively (Table 3). A total of 91,003 ORFs for healthy and 62,136 for disease sample was predicted from the assembled contigs using ORF prediction tool Prodigal.

The EggNOG annotations were assigned to 74,324 predicted ORFs of healthy and 54,077 of disease samples. Out of which 19,575 (healthy) and 17,139 (disease) were assigned with proper GO annotations. The genes were majorly involved in metabolism in both the samples,

Table 2. Alpha diversity indices for the species level taxonomic abundance

Index	Healthy	Diseased
Shannon	6.37	5.48
Simpson	0.95	0.94
Inverse Simpson	18.56	16.90
Fisher Alpha	4411.01	3454.55

Table 3. Metagenome assembly and annotation statistics

Assembler	Diseased	Healthy
Number of contigs	8503	16315
Longest contig (bp)	1304375	719730
N50	12482	5823
L50	695	7627
Min length	2000	2000
Max length	1304375	719730
Average length	6916.87	5092.68
Identified ORFs	62136	91003
Number of GO annotated sequences	17139 / 27.58%	19575 / 21.51%
Number of GO annotations	88195	120172

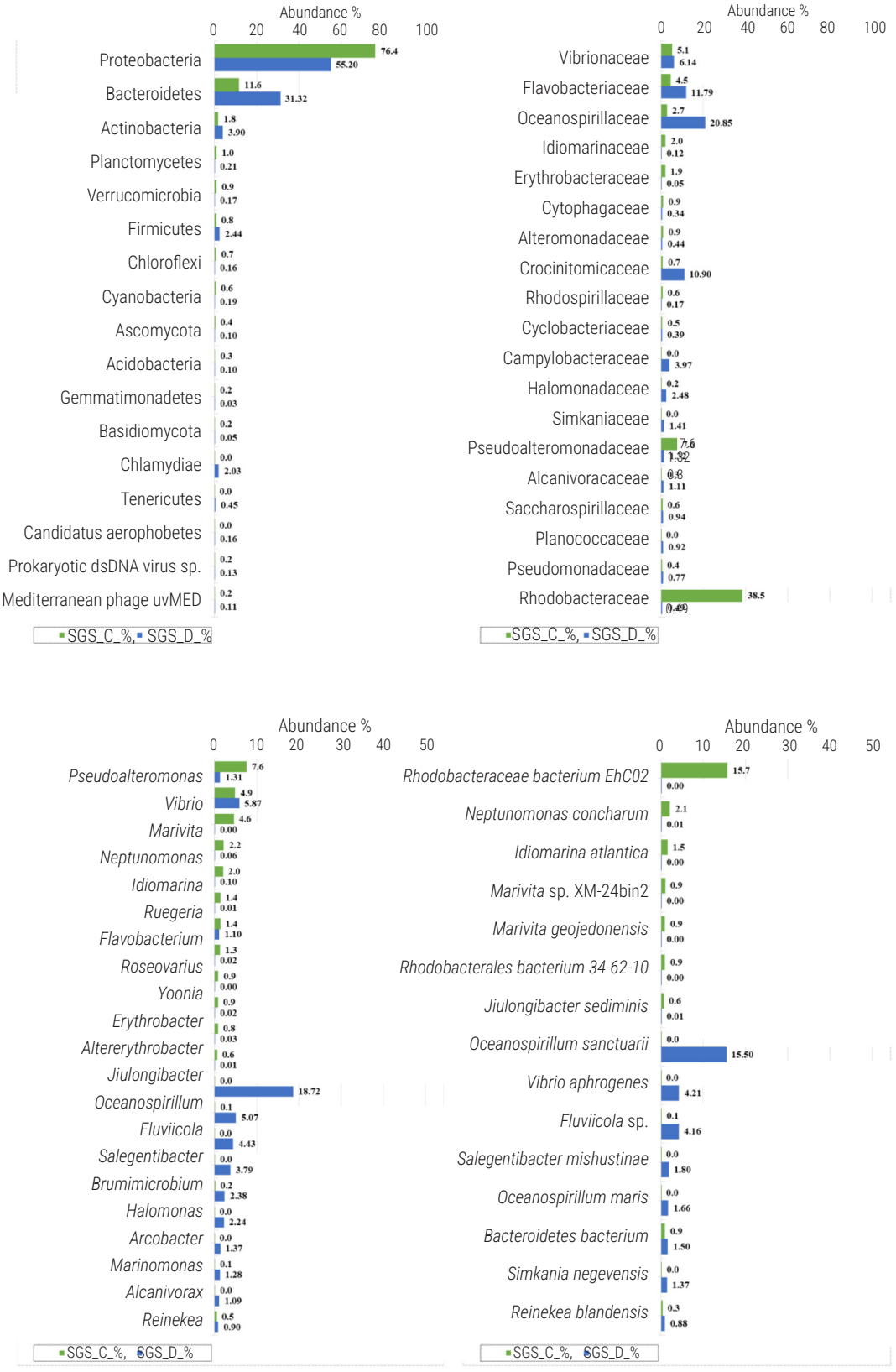

Fig.2: Taxonomic abundances of healthy and diseased samples. (a) Phylum level abundance; (b) Family level abundance; (c) Genus level abundance; (d) Species level abundance

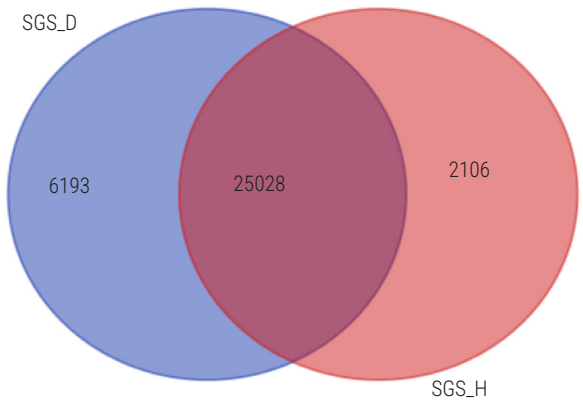

Fig. 3. Venn diagram showing the quantitative taxa composition of the two samples. SGS-H: Healthy; SGS-D: Diseased

especially in amino acid transport and metabolism and energy production and conversion, followed by cellular processing such as defence mechanism, cell wall, membrane and envelope related genes (Fig. 4). Search for antibiotic resistance genes (ARGs) resulted in hits for 147 sequences from healthy sample and 161 sequences from diseased sample (Fig. 5). KEGG pathway map IDs were assigned to 29,055 and 22,510 sequences and toxin-antitoxin systems to 328 and 224 sequences for healthy and diseased samples respectively. In addition, 197 sequences of healthy and 147 sequences of diseased samples were found to be secretion systems genes of type I, II, III, IV, VI and VIII.

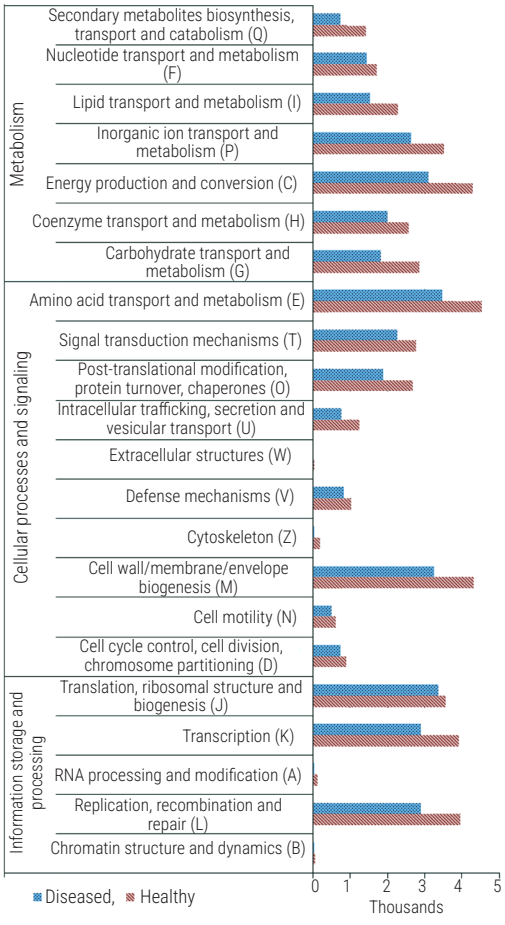

Fig. 4. Distribution of COG categories among healthy and disease samples

Virulence factors

ORFs with minimum 40% sequence coverage and 80% identity against VFDB sequences were considered as virulent genes. A total of 451 putative virulent genes were identified in the healthy sample out of which 42 ORFs showed an identity of 80% and a coverage percentage of 100. Whereas the ORFs from the diseased sample revealed a total of 169 putative virulent genes in which 58 sequences are with 100% query coverage and 80% identity. Number of virulent genes were found to be proportional to the number of ORFs in the samples and did not exhibit any association with disease.

Discussion

Optimal quality of the rearing water is essential for proper growth and development of aquacultured animals, which if not managed properly, would act as a source for exerting biotic or abiotic stress and subsequently cause disease (Li *et al.,* 2021). The composition and stability of microbial communities present in the pond waters are considered to play a major role in disease manifestations (Tello *et al.,* 2020). In the case of shrimp, microbes present in pond waters enter through injury, feed, gills, mouth and antennal gland (Aguirre-Guzman *et. al.,* 2010, Liu *et al.,* 2021) and may act beneficial or detrimental to the host. In recent years, alterations in microbial composition were reported to be the cause of several diseases, however, there are no reports on stunted growth syndrome as on date. Here, we conducted whole metagenomic profiling for two different shrimp ponds in which one of them was infected with stunted growth syndrome. The absence of known disease-causing pathogens (OIE-listed pathogens) like WSSV, IHHNV and EHP, commonly reported to cause growth retardation necessitated this study.

Results revealed *Proteobacteria*, *Bacteroidetes, Actinobacteria* and *Firmicutes*, the commonly reported dominant phyla in the aquaculture environment (Fan and Li, 2019; He *et al.,* 2020), were found to be dominant in both healthy and infected samples. Families *viz., Rhodobacteraceae* and *Pseudoalteromonadaceae* were dominant in the healthy sample, whereas *Oceanospirillaceae, Flavobacteriaceae* and *Vibrionaceae* were dominant in diseased condition. A recent study on white faeces syndrome reported abundance of *Rhodobacteraceae* in healthy shrimps compared to slow-growing shrimps and their surroundings (Wang *et al.,* 2020). *Flavobacteriaceae* and *Rhodobacteraceae* were reported to be potential taxonomic indicators of shrimp health (Xiong *et al.,* 2017). *Oceanospirillum* is one of the common inhabitants of marine environments (Leonard *et al.,* 2000). It has been identified to be potentially pathogenic to pacific white shrimp, oysters and humans (Ostrensky *et al.,* 2018; Horodesky *et al.,* 2020; Zhang *et al.,* 2021). The species-level search revealed differences between healthy and disease samples. The healthy sample showed a high abundance of *Rhodobacteraceae bacterium* EhC02, while the diseased sample was dominated by *Oceanospirillum sanctuarii, Oceanospirillum maris, Fluvicola* sp. and *Vibrio aphrogenes*.

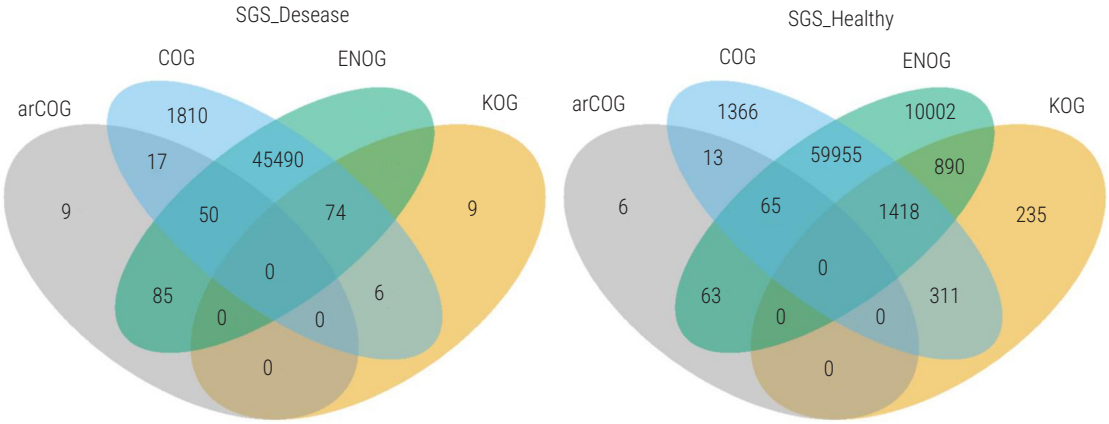

Fig. 5. Venn diagram displaying the number of genes annotated in each category, from the EggNOG annotation pipeline. COG: Clusters of Orthologous Genes, arCOG: Archaeal COG,ENOG: Encyclopedia of Nearby Orthologous Groups, KOG: Eukaryotic Orthologous Groups

Several studies reported lower microbial diversity in disease states in comparison with healthy groups. Here, we observed a similar pattern with regard to alpha diversity indices of SGS samples. This provides empirical evidence for the beneficial effects of more diverse microbial colonies in the habitat. The functional profile revealed that genes related to transcriptional regulators, major facilitator superfamily (MFS), proteins conserved in bacteria, acetyltransferase and ABC transporters were found among the top 10 abundant genes in both the samples. In addition to these, few antibiotic resistance-related genes such as resistance nodulation cell division gene were also observed. Here again, the EggNOG annotation revealed that the functional profile of the diseased sample was associated more with *Oceanospirillales* and *Vibrionales*.

In conclusion, the ambient microbiota showed considerable difference in the healthy and diseased condition, with variations observed in both taxonomic and functional levels. This dysbiosis in the microbiome and its functional profile may be a contributing factor for the disease. In addition, highly abundant opportunistic bacteria still could be a potential threat when the animal is under stress. Future work with better coverage may provide conclusive results in identifying the causal relationships between microbiota and stunted growth disease. The findings of this study provide insights on taxonomic and functional profiles associated with stunted growth syndrome.

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