JFLS | 2017 | Vol 2(1) | Pp1-8

OPEN ACCESS

Journal of Fisheries and Life Sciences
International Peer-Reviewed Biannual Journal
www.fishlifesciencejournal.com

Research Article

Microbial diversity in Rohu fish gut and inland saline aquaculture sediment and variations associated with next-generation sequencing of 16S rRNA gene

Anuj Tyagi* and Balwinder Singh

College of Fisheries, Guru Angad Dev Veterinary and Animal Sciences University, Ludhiana 141004, Punjab, India

ISSN: 2456-6268

ARTICLE INFO
Received: 03 May 2017
Accepted: 10 June 2017
Available online: 30 June 2017

KEYWORDS
Aquaculture
Microbial diversity
Gut microbiota
Next-generation sequencing
16S rRNA

***CORRESPONDENCE**
anujtyaagi@yahoo.co.in

ABSTRACT

Next-Generation Sequencing (NGS) based 16S rRNA gene metagenomics studies are significantly contributing to understand microbial diversities in relatively unexplored agricultural sub-disciplines. However, experimental variations such as primer selection and polymerase chain reaction (PCR) bias can have great impact on outcomes of these studies. Here, we compared two 16S rRNA variable (V3 and V4) region specific primer pairs for study of microbial diversity in sediment and fish (*Labeo rohita*) gut samples. Clear bias in terms of diversity and community representation was observed in NGS dataset generated from V3 primers, whereas V4 primer showed much better results. Overall diversity in sediment sample was found to be much higher than gut sample. Proteobacteria followed by Verrucomicrobia were most dominant phyla in sediment and fish gut samples. Presence of known cellulose degraders (*Clostridium* spp., *Ruminococcus* spp., *Eubacterium* spp. and *Bacteroides* spp.) and Short Chain Fatty Acid (SCFA) producers (*Faecalibacterium prausnitzii*, *Veillonella* spp. and *Megasphaera* spp.) suggested that gut microbiota played an important role in digestion, physiology, metabolism and health of *L. rohita*. Gut microbiota also harbored potentially fish and human pathogenic bacteria. Very few sequences belonging to known probiotic bacteria (*Bacillus* spp., *Streptococcus* spp. and *Bifidobacterium* spp.) were observed. This study serves as preliminary investigation of microbial diversity in aquaculture environment and impact of primer specific variations on estimated diversity.

© 2017 The Authors. Published by JFLS. This is an open access article under the CC BY-NC-ND license (https://creativecommons.org/licenses/by-nc-nd/4.0).

INTRODUCTION

Study of microbial diversity and their functional capabilities, in specific habitat, plays an important role in understanding more about its microbial ecology and evolution (Martinez-Porchas and Vargas-Albores, 2015). However, these studies had been previously suffering from scientific/technological limitations. Culture dependent methods followed by biochemical bacterial identification, are very much labour intensive. The inability to culture, isolate and biochemically identify large number of microbes from a given sample has been their major limitation. As identification of all the bacterial colonies is not possible, the population representation pattern of microbes in sample is lost. Moreover, viable but nonculturable (VBNC) nature of large numbers of bacteria has been a major obstacle for culture dependent approach (Handelsman, 2004; Streit and Schmitz, 2004). With the advent of Sanger sequencing, PCR amplification followed by cloning and sequencing of 16S rRNA gene became a critical part of taxonomic and phylogenetic characterization of environmental microbial communities. Moreover, direct DNA extraction from environmental samples followed by cloning, functional expression screening and sequencing, was also helpful to offer preliminary insights into functional potential of resident microbial communities. In spite of significant contributions, Sanger sequencing was not able to overcome the issues related to population representation loss, high cost and labour (Thomas *et al.*, 2012).

Aquaculture is the major source of food and employment, worldwide. However, efficient production in any aquaculture system depends on proper input and environment management (Ansal *et al.*, 2013). Frequent use of inorganic and organic fertilizers to maintain abundant supply of natural foods helps in sustaining the optimal fish biomass. These conditions also favour microbial growth,

which in turn play an important role as biomarkers, bioremediators, decomposers and probiotics in aquaculture (Martinez-Porchas and Vargas-Albores, 2015). Moreover, microbial communities in aquaculture environment may have strong interrelationship with the resident microbiota of fishes. Similar to other vertebrates, gut microbiota is expected to play an role in fish health and physiology (Ghanbari *et al.*, 2015). Though, significant progress has been made to study the role of microbes in aquaculture, technical challenges associated with culture based methods and Sanger sequencing still remained a major hindrance.

The arrival of Next-Generation Sequencing (NGS) technologies has resulted in changed scientific approaches in microbial ecology research. The co-evolution of new NGS platforms and software tools has allowed generation of large sequencing datasets at unprecedented speed, accuracy with reduced cost (Metzker, 2010). Parallel generation of millions/billions of sequencing reads by these NGS technologies allows simultaneous identification of dominant and rare microbial communities/functional genes in specific environments (Na *et al.*, 2011; Star *et al.*, 2013). These direct DNA analysis approaches have led to tremendous progress in relatively recent genomic sub-discipline of metagenomics. In spite of massive data output, dominant NGS platforms (Illumina, Roche, Ion) can only generate short sequencing reads ranging from 250 bp to 1000 bp. Thus, NGS based 16S rRNA metagenomics studies rely on PCR amplification of specific 16S rRNA hypervariable regions (V1-V9) by using the universal primers targeting the flanking conserved regions (Bikel *et al.*, 2015). Though several studies targeting different 16S rRNA hypervariable region have been reported (Aravindraja *et al.*, 2013; Milani *et al.*, 2013; Petrof *et al.*, 2013; Wu *et al.*, 2012), variable region and primer specific biases should always be considered before starting a new one. Poor PCR primer performance in the form of positive or negative annealing bias against specific 16S rRNA sequences may have significant influence on microbial diversity in final NGS dataset (Siqueira *et al.*, 2012). Large numbers of primers for 16S rRNA metagenomics are available in literature. Klindworth et al., (Klindworth *et al.*, 2013)*in silico* evaluated 16S rRNA specific 175 primers and 512 primer pairs for selection of "best available" primers pairs for domain *Bacteria* and *Archea.* Thus, preliminary evaluation of various primers should be an important step before planning the large scale metagenomic studies. At present, very few (Hong *et al.*, 2009; Milani *et al.*, 2013; O'Sullivan *et al.*, 2014) experimental studies have been conducted to evaluate the influence of primer pairs on 16S rRNA microbial diversity. Moreover, there is critical lack of information on comparative effect of primer pairs on microbial diversity of field samples. In this study, we compared two V3 and V4 region specific primer pairs for study of microbial diversity in inland saline sediment and fish gut content samples. V3 and V4 regions, amplified from soil and fish gut metagenomic DNA samples, were subjected to NGS followed by diversity analysis to understand the microbial diversity and influence of primers on taxonomic representation in aquaculture samples.

MATERIALS AND METHODS

Sample collection and metagenomic DNA extraction

Sediment sample for this study (designated as AT1) was collected from inland saline aquaculture extensive fish culture pond (area – approximately 0.5 acre) located in Fazilka district of Punjab. Approximately 20 g of sediment from the pond bottom was gently scrapped at three places 3-4ft away from each other, pooled in a sterile polypropylene container and transported to laboratory on frozen gel packs. For collection of fish gut content sample (designated as AT2), Rohu (*Labeo rohita*) weighing approximately 800 g were collected from college farms and euthanized by transferring into ice cold water for 30 minutes. After aseptically opening the abdominal cavity, gut content (foregut onwards) was gently squeezed out into a sterile container. Gut contents of three fishes were pooled together. These samples were stored at -80 ^{o}C until further processing within 48 hrs. Metagenomic DNA from 250 mg of sediment and fish gut content samples was extracted by MOBIO Power Soil DNA Isolation Kit (MOBIO Laboratories Inc., USA), as per supplier`s standard protocol. The extracted DNA was subjected to fluorometric quantification using Qubit dsDNA BR Assay Kit (Thermo Fisher Scientific, USA) followed by electrophoresis on 1% agarose gel.

16S rRNA amplicon library preparation and sequencing

Partial gene sequences corresponding to V3 and V4 regions of 16S rRNA gene were PCR amplified from extracted DNA samples. For amplification of V3 region Probio_Uni/Probio_Rev primer pair (Milani *et al.*, 2013), henceforth referred as V3 primer pair, was used. V4 region was amplified by 520F/802R (S-D-Bact-0564-a-S-15/S-D-Bact-0785-b-A-18) primer pair, henceforth referred as V4 primer pair. This V4 primer pair was also selected as one of the most recommended ones for NGS based sequencing of 16S rRNA (Klindworth *et al.*, 2013). The 5` end of each forward primer was fused with Ion Torrent specific A adapter and 10 bases long unique barcode, whereas each reverse primer contained Ion Torrent specific truncated P1 (trP1) adapter at its 5` end. These modifications were carried out in accordance with Ion amplicon library preparation (fusion method) protocol. The 25 µl PCR reaction consisted of 12.5µlAmpliTaq Gold 360 master mix, 0.2 µM of each V3 or V4 primer and 10 - 20 ng of metagenomic DNA. The PCR thermal cycler program consisted of initial denaturation at 95 ^{o}Cat followed by 35 cycles of 94^{o}C for 30 s, 65^{o}C (for V3 primer)/55 ^{o}C (for V4 primer) for 30 s and 72 ^{o}C for 90 s. The final extension step was performed at 72 ^{o}C for 10 min. For V3 primer, the previously reported annealing temperature of 55 ^{o}C(Milani *et al.*, 2013)resulted in nonspecific amplification and generation of multiple amplicon bands. Thus, we had to use the higher annealing temperature of 65 ^{o}C for this particular primer set. After amplification, PCR reaction was cleaned by Agencourt AM Pure XP Beads (Beckman Coulter, Germany) and purified amplicons were quantified by High Sensitivity DNA chip on Agilent 2100 Bioanalyzer. Amplicon libraries for each variable region, containing unique barcodes, were pooled together in equimolar ratios for clonal amplification (emulsion PCR) followed by multiplexed sequencing on Ion S5 system (Thermo Fisher Scientific, India). Preliminary QC of sequencing data was performed by Torrent Suite software (Thermo Fisher Scientific, India) to removal poor quality, polyclonal reads as well as to trim any available adaptor sequences at 3` end.

Sequencing data passing these quality steps was demultiplexed and exported as fastq files.

Analysis of NGS data

For each sample, two sequencing datasets corresponding to V3 and V4 regions were generated to compare the influence of primer pair on estimated microbial community structure and diversity. The fastqfiles were processed by QIIME bioinformatics pipeline (Caporaso *et al.*, 2010). Split_library script was used to parse sequences meeting the pre-defined quality (sequence length > 150 bp for V3 region, sequence length > 200 bp for V4 region, average quality score ≥ 25, homopolymer length ≤ 6 bp, mismatches in primer regions ≤ 2, trimming of any reverse primer sequence) criteria as well as to format the data for downstream processing. These quality filtered sequences were subjected to open reference Operational Taxonomic Units (OTUs) picking strategy by first clustering them against Greengenes reference database (ftp://greengenes.microbio.me/greengenes_release/gg_13_5/gg_13_8_otus.tar.gz) at 97% similarity. Sequences failing to hit the reference database were further clustered *de novo* by uclust at 97% similarity. From each otu cluster, one representative sequence was picked up and subjected to lowest possible taxonomic rank assignment by BLAST method against the Greengenes reference database. The OTU table was converted to text and graphical formats to determine the microbial communities' representation at specific taxonomic levels. To determine whether sequencing data was enough to capture all possible OTUs within the sample, rarefaction plot was generated the by plotting the alpha diversity metrics against number of sequencing reads for rarefied OTU tables. Alpha (within-sample) diversity metrics such as observed otus, Shannon and Simpson index as well as beta (between-samples) diversity weighted unifrac metrics were also calculated to evaluate any primer pair specific variations in estimated microbial compositions.

RESULTS AND DISCUSSION

Sequencing and data QC

A total of 1,309,813 Ion quality approved raw sequencing reads, corresponding to 4 datasets (two for each sample), were generated during the multiplexed sequencing run. Sequence data was submitted to NCBI Short Read Archive (accession number SRP093602). Maximum numbers of reads were generated for V3 region of sample AT1, whereas lowest numbers of reads were obtained for V4 region of sample AT2 (Table 1).

Table 1: Sample and primer specific sequencing reads and diversity indexes

Sample	Primer Pair	Dataset Name	Sequencing Reads (Raw)*	Reads Passing QC**	Observed OTUs	Observed Phyla	Shannon Index	Simpson Index
AT1	V3	AT1V3	549988	209812	1473	11	5.09	0.89
	V4	AT1V4	333307	147537	4874	62	9.78	0.99
AT2	V3	AT2V3	364068	182482	487	8	4.53	0.87
	V4	AT2V4	62450	28793	804	23	6.24	0.94
		AT1V3V4[#]	883295	368361	6679	64	8.24	0.96
		AT2V3V4[#]	426518	213241	1385	23	5.84	0.93

*Ion quality approved sequencing reads exported from Torrent Suite Software

**Reads passing the QIIME QC criteria

[#]For generation of dataset AT1V3V4, fastq files AT1V3 and AT1V4 were merged together followed by QIIME QC and downstream data analysis. Dataset AT2V3V4 was also generated in similar fashion.

QIIME quality filtering led to removal of 49.88 – 61.85% of raw sequencing reads. Previous studies have also shown that large fraction of sequencing reads get removed during the QC step(Milani *et al.*, 2013).Among the failed reads in our data, majority (56.63 - 81.10%) got filtered out due to failure of primer mismatch criteria. During the fastqc analysis of demultiplexed fastq files, initial sequencing region of 20 bp corresponding to forward V3/V4 primer showed a dip in quality scores (data not shown). As per our understanding, this surprising dip during sequencing of primer region could be behind the high numbers of reads failing to satisfy the primer mismatch of ≤2 bases.

Assessment of sequencing depth and microbial diversity

In this study, 7396 OTUs were obtained from a total of 581,602 QIIME QC filtered sequencing reads belonging to four datasets. Combined analysis of both the variable regions for each sample revealed that inland saline sediment sample AT1 (dataset AT1V3V4) with 6,679 OTUs (64 Phyla), had much higher diversity than fish gut sample AT2 (dataset AT2V3V4) with 1,385 OTUs (23 Phyla). Higher values of Shannon and Simpson indexes for sample AT1 also supported this observation (Table 1). Several studies have previously reported high microbial diversities in freshwater and marine aquaculture sediments (Aravindraja *et al.*, 2013; Bissett *et al.*, 2006; Wu *et al.*, 2012). Some of these studies also indicated that actual microbial diversity in sediment samples could be much higher than experimentally detected. Gut content of several herbivorous and planktivorous carps has also been analyzed leading to discovery of 259 to 2325 OTUs in these fishes (Li *et al.*, 2014; Li *et al.*, 2015; Wu *et al.*, 2012). However, little information is available on comparative diversities of microbial communities in aquatic animals and surrounding environment. Wu and colleagues (Wu *et al.*, 2012) studied the microbial diversity in freshwater aquaculture sediment and gut content of grass carp (*Ctenopharyngodon idellus*) by pyrosequencing of 16S rRNA gene fragments. Sediment sample had higher microbial diversity than gut content. A similar study also indicated higher bacterial diversity in surrounding water sample than in gut of Chinese mitten crab (Zhang *et al.*, 2016). These observations suggest that harsh

gut environment supports the growth and dominance of very specific microbial groups leading to less microbial diversity.

In our study, Influence of primer specific variations was clearly observed on estimated diversities. For each sample, numbers of OTUs detected in V4 dataset were considerably higher than V3 dataset, even though the numbers of sequencing reads in V4 datasets were quite lower than V3 datasets (Table 1). During beta diversity analysis, weighted UniFrac Principle Coordinate Analysis (PCoA) plot showed primer-wise clustering of datasets rather than expected sample-wise clustering (Fig. S1). Venn diagram also suggested that very few OTUs were shared between V3 and V4 datasets of each sample (Fig. 1). These observations clearly indicated that V3 and V4 primer pairs targeted almost entirely different sets of bacterial communities in metagenomic DNA. These results reinforce the observations that optimum PCR plays an important role in 16s rRNA amplicon based metagenomic studies and any amplification bias may have significant impact on estimated diversity (Bikel *et al.*, 2015; Kuczynski *et al.*, 2011) . It has been reported that poor sequencing depth may also result in underestimation of microbial diversity (Bissett *et al.*, 2006). In this study, rarefaction curves for all the datasets approached the plateau, suggesting that enough sequencing depth was achieved for each one (Fig. 2).

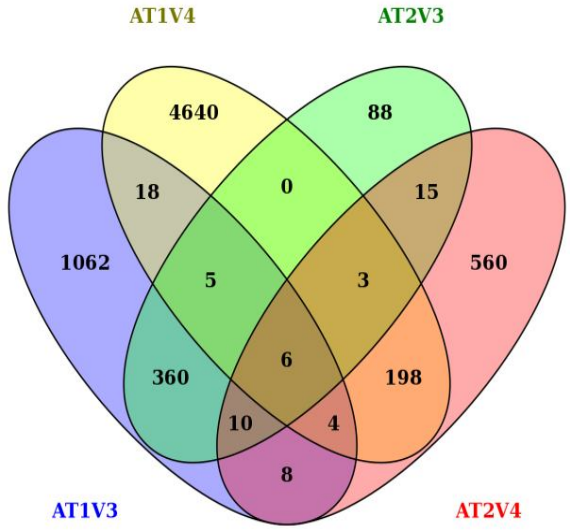

Fig.1: Venn diagram analysis of shared OTUs. Very less number of OTUs were found to be common between different primer sets of same sample.

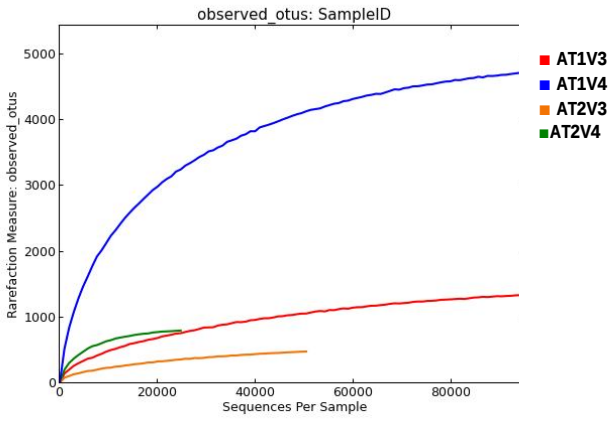

Fig. 2 Rarefaction analysis. For all the datasets, observed OTUs counts approached the plateau.

Taxonomic composition of sediment and gut microbiota

Based on our own findings and previous observations that PCR primers can have significant impact on estimated community structure, we decided to separately analyze the taxonomic composition for each sample-primer sequencing dataset (Fig. 3, Supplementary Krona Charts S2-S5). For sediment sample AT1, very few phyla (11) were detected (Table 1) in V3 dataset (AT1V3). Among all these phyla, Bacteroidetes was most dominant with 96.2% reads belonging to this particular phylum. *Prevotella copri* with 72% reads was found to be most dominant organism in Bacteroidetes. However, V4 dataset (AT1V4) reported much diverse taxonomic compositions with detection of 62 phyla and it was only used to further determine the taxonomic representation in sediment sample. Among all these phyla, Proteobacteria was most dominant (41%) followed by Verrucomicrobia (11%) and OD1 (11%). Other important phyla like Acidobacteria, Chloroflexi, Tenericutes and Firmicutes comprised of 4-5% sequencing reads for each. Surprisingly, this particular dataset had very few sequences (0.4%) belonging to Bacteroidetes. Wang and colleagues (Wang *et al.*, 2012) studied the bacterial diversities in freshwater, intertidal wetland, and marine sediments Illumina by using 16S rRNA tags. In all the samples, Proteobacteria were found to be most dominant representing up to 60% of all phyla. Other important phyla were found to be Nitrospira, Verrucomicrobia, Acidobacteria and Firmicutes. Another study of marine sediment also revealed dominance of Firmicutes followed by Proteobacteria (Kochling *et al.*, 2011). Microbial diversity studies, specifically carried out in aquaculture sediments, have also pointed towards dominance of Proteobacteria (Bissett *et al.*, 2006; Wu *et al.*, 2012). One common trend among all these studies was much less (as low as 3%) abundance of Bacteroidetes in comparison to Proteobacteria. Among the phylum Proteobacteria in V4 dataset of sediment sample (AT1V4), Deltaproteobacteria (45%), Gammaproteobacteria (26%) and Alphaproteobacteria (23%) were the dominant classes. Strictly anaerobic sulphur (*Desulfuromonas* spp.)and sulphate-reducing (*Desulfotignum* spp., *Desulfococcus* spp., *Desulfovibrio* spp.) bacteria were found to be dominant in class Deltaproteobacteria. These organisms play an important role in biogeochemical cycles through the oxidation of inorganic and organic carbon (Muyzer and Stams, 2008). Sulphate-reducing bacteria can also play a role in nitrogen fixation by controlling the release of phosphate leading to creation of reduced environment (Howarth *et al.*, 1988). Among the class Alphaproteobacteria, order Rhodobacterales, Rhodospirillales contain chemoorganotrophs and photoheterotrophs as well as nitrogen fixing *Azospirillum* spp. The order Rhizobiales contains many nitrogen fixing plant-symbiotic bacteria and photoautotrophs (Caumette *et al.*, 2015). Overall a rich diversity and abundance of Alphaproteobacteria was observed in sediment sample. Previous studies have also indicated that diversity and abundance of Alphaproteobacteria in aquatic ecosystem is dependent on its primary productivity (Claire Horner-Devine *et al.*, 2003).

Similar to sediment sample, V3 dataset (AT2V3) for fish gut content also showed dominance of Bacteroidetes with 96.7% reads belonging to this particular phylum.

However, V4 dataset (AT2V4) showed presence of large numbers (23) of phyla in fish gut. The 804 OTUs in this datasets belonged to 215 distinct taxonomic groups during species level taxonomic assignment (Supplementary Excel Sheet S6). During the human gut microbiome study, prevalence of 1,000 to 1,150 bacterial species was determined (Qin *et al.*, 2010).

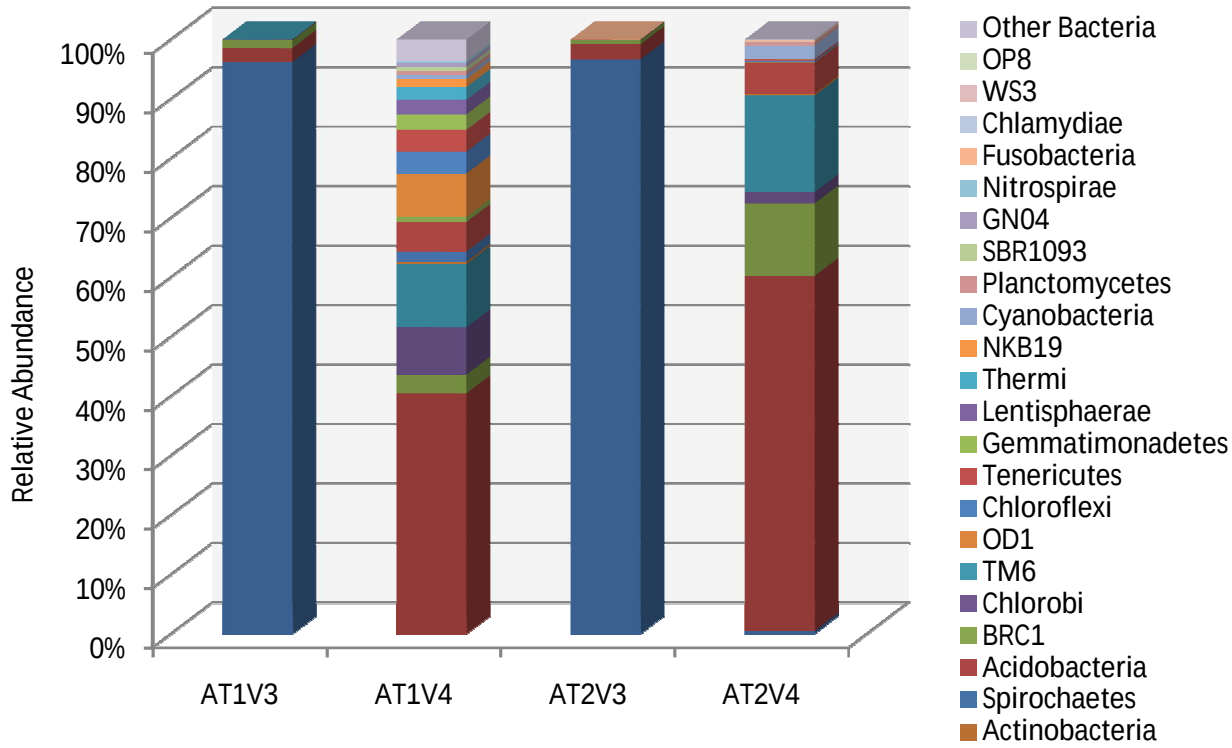

Fig. 3 Taxonomic compositions of bacterial communities. Relative abundance of up to 20 top most phyla was plotted. Rest of the phyla were grouped into other bacteria category.

We proposed that actual species count in fish gut could be much higher than our reported value of 215, as many reads in our study could be assigned only up to family or genus levels. As per this dataset, Proteobacteria (61%), followed by Verrucomicrobia (16%), Firmicutes (13%) and Acidobacteria (5%) were found to be dominant phyla. Proteobacteria and Firmicutes are the important members of intestinal microbiota and several studies have reported their dominance in gut of carps and other fishes (Han *et al.*, 2010; Wu *et al.*, 2012; Xia *et al.*, 2014; Xing *et al.*, 2013). On the other hand, Bacteroidetes have been found relatively less abundant (<0.4 – 8.2%) in fish gut (Xia *et al.*, 2014; Xing *et al.*, 2013). We also had low prevalence (0.5%) of Bacteroidetes in our V4 gut dataset. These observations suggest that PCR bias in V3 primer is leading to artificial overestimation of Bacteroidetes in soil and gut samples. Thus, we further used only V4 primer dataset (AT2V4) to understand the relative microbial community representation in fish gut. It is now well established that taxonomic composition and interactions of resident microbiota with host play an important role in its physiology, nutrition and health. Over the last few years, gut microbiome studies have been major point of focus not only to understand more about pathogenic and probiotic bacterial groups but also to get precious insights about the resident microbes capable of efficient digestion and metabolism of dietary substances (Xia *et al.*, 2014). In aquaculture system, both indigenous and introduced pathogens can cause diseases and their occurrence depends on their ecology, source and survival capabilities. These bacterial species can be facultative pathogenic for both fish and human beings and may be isolated from fish without apparent symptoms of the disease (Novotny *et al.*, 2004). Large number of pathogenic bacteria belongs to Gammaproteobacteria and this particular class was found to be quite abundant in sediment and fish gut samples. In case of fish gut, Gammaproteobacteria were dominated by Xanthomonadales (78%) and other bacterial groups were found to relatively less abundant (≤2%). However, distribution in sediment sample was found to be much different with Xanthomonadales, Oceanospirillales, Alteromonadales, Chromatiales, Thiotrichales forming 6-21% of Gammaproteobacteria followed by less abundant groups. Several food-borne human pathogens belong to family Enterobacteriaceae. This particular family was found to be very less prevalent in sediment as well as in fish gut samples and majority of reads could not be classified below family level. Deeper taxonomic classification of partial sequence fragments has been a major challenge in 16S rRNA based metagenomics studies. During analysis of V3-V5 pyrosequencing datasets, it was reported that 10-29% of reads could not be classified at genus level. Among these unassigned reads, majority (63-83%) were from Enterobacteriaceae family (Plummer *et al.*, 2015). Several species of *Aeromonas*, *Pseudomonas*, *Vibrio* and *Flavobacterium* are important fish pathogens as well as opportunistic human pathogens. In our study, very low (<0.4%) presence of these genera was found in sediments and fish gut samples. These results are in agreement with previous reports (Wu *et al.*, 2012). In addition, sequences

belonging to family Peptostreptococcaceae, a potentially human pathogenic group, were also found in fish gut.

It is now well known that gut microbiota plays an important role in digestion of complex dietary substances as well as in maintaining the health status of host. Similar to human (David *et al.*, 2014), taxonomic composition of fish gut microbiota also quickly (within days) responds to changes in dietary habits and health conditions of host (Li *et al.*, 2014; Xia *et al.*, 2014). As Juveniles and adults of *L. rohita* have strong preference for phytoplanktons and macro-vegetation, the role of microbes in degradation of complex polysaccharides needs deeper investigations. Firmicutes are known to be dominant gut microbiota of human and other terrestrial animals. Several bacterial species in this phylum can degrade insoluble complex carbohydrates to simple soluble ones (Qin *et al.*, 2010), which are further used as a source of energy for host and other gut microbial communities. Among the Firmicutes, members of family Clostridiaceae were found to be most abundant (48%) and few of these reads also belonged to *Clostridium prefringens*. *Clostridium* is obligate anaerobic bacteria and some of its species (*C. botulinum, C. difficile and C. prefringens*) are important human pathogens. However, several species of *Clostridium* are known to have effective cellulase systems for complete degradation of cellulose to glucose (Flint *et al.*, 2012). This observation along with dietary habit of *L. rohita* explains the relatively high abundance of *Clostridium* in gut. Several others potentially cellulose degrading Firmicutes (*Ruminococcus* spp., *Eubacterium* spp.) and non-Firmicutes (*Bacteroides* spp.) were also observed in fish gut. In complex gut environment, glucose may not always be the final product of cellulose degradation and its bacterial fermentation may lead to production of Short Chain Fatty Acids (SCFAs) such as acetate, propionate and butyrate (den Besten *et al.*, 2013). In our study, sequences belonging to *Faecalibacterium prausnitzii*, a butyrate producer, were observed. Moreover, some propionate producers (*Veillonella* spp., *Megasphaera* spp.) belonging to family Veillonellaceae (Flint *et al.*, 2015) were also detected in fish gut. In mammals, large fraction of SCFAs is absorbed in gut where it not only provides the energy to colonic mucosa but also have anti-inflammatory and anti-apoptotic effects (Flint *et al.*, 2012). Moreover, SCFAs have antimicrobial activity against pathogenic bacteria and they have also been used in commercial diets to control *Salmonella*(Van Immerseel *et al.*, 2002). Antibacterial activity of SCFAs against luminescent vibrios has also been reported (Defoirdt *et al.*, 2006). Thus, SCFAs play an important role in maintaining the health of gastrointestinal epithelium. In our study, presence of SCFAs producing microbiota in fish gut is a significant observation. The modulation of these SCFAs producing microbes could be an important area of nutrition research to improve overall fish health, metabolism and growth. Some of the Firmicutes such as *Bacillus*, *Lactobacillus* and *Streptococcus* have often been used as probiotics in aquaculture. In this study, very low (3% of Firmicutes) abundance of *Bacillus* was noticed in gut microbiota. Few sequences related to order Lactobacillales were found in gut dataset but these ultimately matched with *Streptococcus* spp./*S. alactolyticus* during lower taxonomic assignment. Actinobacteria is a key phylum in human gut and several of its species have the ability to produce secondary metabolites such as antibiotics. It is now well known that some Actinobacteria belonging to *Bifidobacterium* spp. play an important role maintaining the host health and they have also been used as commercial probiotics for human use (O'Callaghan and van Sinderen, 2016). In our study Actinobacteria were found to be much less abundant (0.3%) and majority (60%) of these belonged to *Bifidobacterium* spp./*B. adolescentis*/*B. bifidum*. Thus, our findings are in agreement with Wu and colleagues (Wu *et al.*, 2012) that probiotic bacteria are unable to establish large populations in fish intestine.

This study provides much desired information about microbial diversity in aquaculture and fish gut environment and associated experimental variations. Clear bias in terms of diversity and community representation was observed in NGS dataset generated from V3 primers, whereas V4 primer showed much better results. Diversity in sediment sample was found to be higher than fish gut sample. Proteobacteria followed by Verrucomicrobia were most dominant phyla in sediment and fish gut samples. Presence of known cellulose degraders and Short Chain Fatty Acid (SCFA) producers suggested that gut microbiota played an important role in digestion, physiology, metabolism and health of *L. rohita*. Gut microbiota also harbored potentially fish and human pathogenic bacteria. We suggest that preliminary evaluation of experimental variation should be carried out before starting a large scale study. Our finding could be used as baseline data to carry more detailed studies in various physiological and geographical conditions.

ACKNOWLEDGMENT

The authors are grateful to the Dean (College of Fisheries, Guru Angad Dev Veterinary & Animal Sciences University, Ludhiana, India) for all necessary support during this study. Excellent lab and data analysis support provided by Dr. Neeraj Chauhan, Mr. Pravin Nilawe, Mr. Hersh Parikh (Thermo Fisher Scientific, India) is also acknowledged.

Metagenomics research in our lab is supported by Science & Engineering Research Board, Department of Science & Technology (DST-SERB) Young Scientist Start Up Research Grant (YSS/2014/000269). The DST-SERB funding support for this work is duly gratefully acknowledged.

REFERENCE

Ansal, M.D., Dhawan, A., Singh, G. 2013. Productivity of freshwater carps in inland salt affected waterlogged areas of Punjab, India - A field study. *Indian J. Ecol.* 40(2): 284-289.

Aravindraja, C., Viszwapriya, D., Karutha Pandian, S. 2013. Ultradeep 16S rRNA sequencing analysis of geographically similar but diverse unexplored marine samples reveal varied bacterial community composition. *PLoS One* 8(10): e76724.

Bikel, S., Valdez-Lara, A., Cornejo-Granados, F., Rico, K., Canizales-Quinteros, S., Soberon, X., Del Pozo-Yauner, L., Ochoa-Leyva, A. 2015. Combining metagenomics, metatranscriptomics and viromics to explore novel microbial interactions: towards a systems-level understanding of human microbiome. *Comput Struct Biotechnol J* 13: 390-401.

Bissett, A., Bowman, J., Burke, C. 2006. Bacterial diversity in organically-enriched fish farm sediments. *FEMS Microbiol. Ecol.* 55(1): 48-56.

Caporaso, J.G., Kuczynski, J., Stombaugh, J., Bittinger, K., Bushman, F.D., Costello, E.K., Fierer, N., Pena, A.G., Goodrich, J.K., Gordon, J.I., Huttley, G.A., Kelley, S.T., Knights, D., Koenig, J.E., Ley, R.E., Lozupone, C.A., McDonald, D., Muegge, B.D., Pirrung, M., Reeder, J., Sevinsky, J.R., Turnbaugh, P.J., Walters, W.A., Widmann, J., Yatsunenko, T., Zaneveld, J., Knight, R. 2010. QIIME allows analysis of high-throughput community sequencing data. *Nat. Methods* 7(5): 335-336.

Caumette, P., Brochier-Armanet, C., Normand, P. 2015. Taxonomy and Phylogeny of Prokaryotes. In: Bertrand, J.-C., Caumette, P., Lebaron, P., Matheron, R., Normand, P., Sime-Ngando, T. (Eds.), Environmental Microbiology: Fundamentals and Applications: Microbial Ecology. Springer Netherlands, Dordrecht, pp. 145-190.

Claire Horner-Devine, M., Leibold, M.A., Smith, V.H., Bohannan, B.J.M. 2003. Bacterial diversity patterns along a gradient of primary productivity. *Ecol. Lett.* 6(7): 613-622.

David, L.A., Maurice, C.F., Carmody, R.N., Gootenberg, D.B., Button, J.E., Wolfe, B.E., Ling, A.V., Devlin, A.S., Varma, Y., Fischbach, M.A., Biddinger, S.B., Dutton, R.J., Turnbaugh, P.J. 2014. Diet rapidly and reproducibly alters the human gut microbiome. *Nature* 505(7484): 559-563.

Defoirdt, T., Halet, D., Sorgeloos, P., Bossier, P., Verstraete, W. 2006. Short-chain fatty acids protect gnotobiotic Artemia franciscana from pathogenic Vibrio campbellii. *Aquaculture*, 261(2): 804-808.

den Besten, G., van Eunen, K., Groen, A.K., Venema, K., Reijngoud, D.J., Bakker, B.M. 2013. The role of short-chain fatty acids in the interplay between diet, gut microbiota, and host energy metabolism. *J. Lipid Res.* 54(9): 2325-2340.

Flint, H.J., Duncan, S.H., Scott, K.P., Louis, P. 2015. Links between diet, gut microbiota composition and gut metabolism. *Proc Nutr Soc* 74(1): 13-22.

Flint, H.J., Scott, K.P., Duncan, S.H., Louis, P., Forano, E. 2012. Microbial degradation of complex carbohydrates in the gut. *Gut Microbes* 3(4): 289-306.

Ghanbari, M., Kneifel, W., Domig, K.J. 2015. A new view of the fish gut microbiome: advances from next-generation sequencing. *Aquaculture* 448: 464-475.

Han, S., Liu, Y., Zhou, Z., He, S., Cao, Y., Shi, P., Yao, B., Ringø, E. 2010. Analysis of bacterial diversity in the intestine of grass carp (Ctenopharyngodon idellus) based on 16S rDNA gene sequences. *Aquacult. Res.* 42(1): 47-56.

Handelsman, J. 2004. Metagenomics: application of genomics to uncultured microorganisms. *Microbiol. Mol. Biol. Rev.* 68(4): 669-685.

Hong, S., Bunge, J., Leslin, C., Jeon, S., Epstein, S.S. 2009. Polymerase chain reaction primers miss half of rRNA microbial diversity. *ISME J* 3(12): 1365-1373.

Howarth, R.W., Marino, R., Cole, J.J. 1988. Nitrogen fixation in freshwater, estuarine, and marine ecosystems. 2. Biogeochemical controls. *Limnol. Oceanogr.* 33(4): 688-701.

Klindworth, A., Pruesse, E., Schweer, T., Peplies, J., Quast, C., Horn, M., Glockner, F.O. 2013. Evaluation of general 16S ribosomal RNA gene PCR primers for classical and next-generation sequencing-based diversity studies. *Nucleic Acids Res.* 41(1): e1.

Kochling, T., Lara-Martin, P., Gonzalez-Mazo, E., Amils, R., Sanz, J.L. 2011. Microbial community composition of anoxic marine sediments in the Bay of Cadiz (Spain). *Int. Microbiol.* 14(3): 143-154.

Kuczynski, J., Lauber, C.L., Walters, W.A., Parfrey, L.W., Clemente, J.C., Gevers, D., Knight, R. 2011. Experimental and analytical tools for studying the human microbiome. *Nat. Rev. Genet.*13(1): 47-58.

Li, J., Ni, J., Li, J., Wang, C., Li, X., Wu, S., Zhang, T., Yu, Y., Yan, Q. 2014. Comparative study on gastrointestinal microbiota of eight fish species with different feeding habits. *J. Appl. Microbiol.* 117(6): 1750-1760.

Li, T., Long, M., Gatesoupe, F.J., Zhang, Q., Li, A., Gong, X. 2015. Comparative analysis of the intestinal bacterial communities in different species of carp by pyrosequencing. *Microb. Ecol.* 69(1): 25-36.

Martinez-Porchas, M., Vargas-Albores, F. 2015. Microbial metagenomics in aquaculture: a potential tool for a deeper insight into the activity. *Reviews in Aquaculture* 7: 1-15.

Metzker, M.L. 2010. Sequencing technologies - the next generation. *Nat. Rev. Genet.* 11(1): 31-46.

Milani, C., Hevia, A., Foroni, E., Duranti, S., Turroni, F., Lugli, G.A., Sanchez, B., Martin, R., Gueimonde, M., van Sinderen, D., Margolles, A., Ventura, M. 2013. Assessing the fecal microbiota: an optimized ion torrent 16S rRNA gene-based analysis protocol. *PLoS One* 8(7): e68739.

Muyzer, G., Stams, A.J. 2008. The ecology and biotechnology of sulphate-reducing bacteria. *Nat. Rev. Microbiol.* 6(6): 441-454.

Na, H., Kim, O.S., Yoon, S.H., Kim, Y., Chun, J. 2011. Comparative approach to capture bacterial diversity of coastal waters. *J Microbiol* 49(5): 729-740.

Novotny, L., Dvorska, L., Lorencova, A., Beran, V., Pavlik, I. 2004. Fish: a potential source of bacterial pathogens for human beings. *Vet. Med. – Czech* 49: 343–358.

O'Callaghan, A., van Sinderen, D. 2016. Bifidobacteria and Their Role as Members of the Human Gut Microbiota. *Frontiers in Microbiology* 7(925).

O'Sullivan, D.M., Laver, T., Temisak, S., Redshaw, N., Harris, K.A., Foy, C.A., Studholme, D.J., Huggett, J.F. 2014. Assessing the accuracy of quantitative molecular microbial profiling. *Int J Mol Sci* 15(11): 21476-21491.

Petrof, E.O., Gloor, G.B., Vanner, S.J., Weese, S.J., Carter, D., Daigneault, M.C., Brown, E.M., Schroeter, K., Allen-Vercoe, E. 2013. Stool substitute transplant therapy for the eradication of Clostridium difficile infection: 'RePOOPulating' the gut. *Microbiome* 1(1): 3.

Plummer, E., Twin, J., Bulach, D.M., Garland, S.M., Tabrizi, S.N. 2015. A Comparison of Three Bioinformatics Pipelines for the Analysis of Preterm Gut Microbiota using 16S rRNA Gene Sequencing Data. *J Proteomics Bioinform* 8: 283-291.

Qin, J., Li, R., Raes, J., Arumugam, M., Burgdorf, K.S., Manichanh, C., Nielsen, T., Pons, N., Levenez, F., Yamada, T., Mende, D.R., Li, J., Xu, J., Li, S., Li, D.,

Cao, J., Wang, B., Liang, H., Zheng, H., Xie, Y., Tap, J., Lepage, P., Bertalan, M., Batto, J.M., Hansen, T., Le Paslier, D., Linneberg, A., Nielsen, H.B., Pelletier, E., Renault, P., Sicheritz-Ponten, T., Turner, K., Zhu, H., Yu, C., Li, S., Jian, M., Zhou, Y., Li, Y., Zhang, X., Li, S., Qin, N., Yang, H., Wang, J., Brunak, S., Dore, J., Guarner, F., Kristiansen, K., Pedersen, O., Parkhill, J., Weissenbach, J., Meta, H.I.T.C., Bork, P., Ehrlich, S.D., Wang, J. 2010. A human gut microbial gene catalogue established by metagenomic sequencing. *Nature* 464(7285): 59-65.

Siqueira, J.F., Jr., Fouad, A.F., Rocas, I.N. 2012. Pyrosequencing as a tool for better understanding of human microbiomes. *J Oral Microbiol* 4.

Star, B., Haverkamp, T.H., Jentoft, S., Jakobsen, K.S. 2013. Next generation sequencing shows high variation of the intestinal microbial species composition in Atlantic cod caught at a single location. *BMC Microbiol.* 13: 248.

Streit, W.R., Schmitz, R.A. 2004. Metagenomics--the key to the uncultured microbes. *Curr. Opin. Microbiol.* 7(5): 492-498.

Thomas, T., Gilbert, J., Meyer, F. 2012. Metagenomics - a guide from sampling to data analysis. *Microb Inform Exp* 2(1): 3.

Van Immerseel, F., Cauwerts, K., Devriese, L.A., Haesebrouck, F., Ducatelle, R. 2002. Feed additives to control Salmonella in poultry. *World's Poultry Science Journal* 58(4): 501-513.

Wang, Y., Sheng, H.F., He, Y., Wu, J.Y., Jiang, Y.X., Tam, N.F., Zhou, H.W. 2012. Comparison of the levels of bacterial diversity in freshwater, intertidal wetland, and marine sediments by using millions of illumina tags. *Appl. Environ. Microbiol.* 78(23): 8264-8271.

Wu, S., Wang, G., Angert, E.R., Wang, W., Li, W., Zou, H. 2012. Composition, diversity, and origin of the bacterial community in grass carp intestine. *PLoS One* 7(2): e30440.

Xia, J.H., Lin, G., Fu, G.H., Wan, Z.Y., Lee, M., Wang, L., Liu, X.J., Yue, G.H. 2014. The intestinal microbiome of fish under starvation. *BMC Genomics* 15(1): 266.

Xing, M., Hou, Z., Yuan, J., Liu, Y., Qu, Y., Liu, B. 2013. Taxonomic and functional metagenomic profiling of gastrointestinal tract microbiome of the farmed adult turbot (Scophthalmus maximus). *FEMS Microbiol. Ecol.* 86(3): 432-443.

Zhang, M., Sun, Y., Chen, L., Cai, C., Qiao, F., Du, Z., Li, E. 2016. Symbiotic Bacteria in Gills and Guts of Chinese Mitten Crab (Eriocheir sinensis) Differ from the Free-Living Bacteria in Water. *PLoS One* 11(1): e0148135.