Original Research Article

The metagenomic characterization of bacterial community in aquacultured Shrimp*Penaeusmonodon*

Abstract

Aquaculture has grown rapidly during the last few decades due to research and developmental activitiesbut we are still unaware of the various microbial species thriving within the aquaculture systems and their specific roles for knowing thatMmetagenomics study requiredenvironmental sampleto discover the unexplored microbial community. The aim of this present study isto characterize the gut microbiota of giant tiger shrimp, *Penaeus monodon*collected gut sample and16S rRNA gene-based high-throughput sequencing revealed distinct and diverse microbial communities.The results showed a high abundance of Betaproteobacteria,followed by Alphaproteobacteria,Clostridia,Actinobacteria,Gammaproteobacteria and Bacillifound inthe gut sampleMicrobes that play essential roles in nutrient cycling and mineralization of organic compounds such as Bacteroidetes, Planctomycetes, Gammaproteobacteria, Firmicutes, Cyanobacteria, and Actinobacteria could also

be identified. Due to the strong influence of the gut microbiota on fish health, dominant bacterial species in the gut are strong candidates for probiotics.These findings provide valuable information on the microbial community and contribute to controlling the diseases in shrimp farms.

Keywords:Metagenomics, Bacterial community,Next generation sequencing,16S rRNA

Introduction

The shortage of wild fishery resources and the rising demand for human nutrition has driven a great expansion in aquaculture during the last decades in terms of production and economic value. However, the intensification of seafood farming has resulted in higher risks of disease outbreaks and in the increased use of antimicrobials to control them. The selective pressure exerted by these drugs provides the ideal conditions for the emergence of antimicrobial resistance hotspots in aquaculture facilities. Aquaculture is the fastest-growing animal food-producing sector and is set to overtake capture fisheries as a source of food fish

(Subasighe 2005). Currently, one of the main factors limiting the expansion and profitability of aquaculture is the lack of disease control (FDA 2012).However, the gut microbiota strongly influences fish health in other ways such as assisting in the development of the gut epithelium, providing essential nutrients and stimulating the innate immune system (Nayak 2010).The use of microorganisms in aquaculture as environmental biomarkers, bioremediation, probiotics, and as a direct food source for the cultured species has expanded further in the last few decades. However, we are still unaware of the various microbial species thriving within the aquaculture systems and their specific roles. Evidence has revealed that the diversity of microorganisms in aquaculture systems is far from being elucidated. Metagenomics is the study of genetic material recovered directly from the environmental sample. It is a culture-independent approach that provides an ample opportunity to discover the unexplored microbial community Metagenomics undoubtedly can provide additional information regarding the understanding of the microbial diversity that thrives within the aquaculture systems. The present study reports metagenomic sequencing and analysis of the sediment samples of a semi-intensive penaeid shrimp culture system to explore its microbial diversity. 16S rRNA gene-based high throughput sequencing was employed to reveal distinct and diverse microbial communities present in the sample.

Material and methods

Sample collection and processing

The present study was carried out in a semi-intensive aquaculture system for Penaeusmonodonproduction,located in southern coastal areas of Kerala, India.The aquaculture system operates under semi-intensive management.Approximately 25 harvested shrimp samples were collected from the culture pond from a depth of 70 cm by using a sterile grab.The samples were transferred to ice baskets.The shrimp gutswere isolated with proper care. Gut DNA isolationwas done at the CEPCI research centre,in Kollam,Kerala.The isolated gutwas immediately transferred to ethanol. Gut DNAextraction wasdone as per the manufacturer's recommendation.Extracted DNA from the samples was subjected to NanoDrop and GEL Check before being taken for further steps. The NanoDrop readings of 260/280 at a ~ value of 1.8 to 2 are used to determine the DNA's quality.

Next generation Sequencing Analysis

Metagenomic nucleic acid extracted from the gut was subjected to 16S rRNA gene-based high throughput sequencing and analysis at PhytocomPharmaceuticals

(P)Ltd,Kalamassery,Kerala, India.PCR Amplification with V3 –V4 Primers.40ng of Extracted DNA is used for amplification along with 10pM of each primer.The amplified 16s PCR Product is purified and subjected to GEL Check and Nanodrop QC.The amplicons from each sample were purified with Ampure beads to remove unused primers and an additional 8 cycles of PCR was performed using Illumina barcoded adapters to prepare the sequencing libraries.Sequencing was performed using IlluminaMiseq with a 2x300PE v3 sequencing kit.Raw data identification is done using FASTQC and MULTIQC, followed by trimming of adapters and low-quality reads by TRIMGALORE.The databases used are SILVA / GREENGENES /NCBI.Each read is classified based on percentage coverage and identity.The kraken-build parameter was used to build databases for the analysis. The source to build the database was downloaded from NCBI.The raw data is trimmed to remove the adapter sequences using the tool Trimgalore version 0.45.The trimmed raw data was used as the input for theKraken 2 analysis.

Results

The microbial diversity is assumed to be greater in aquaculture systems due to the presence of nitrogenous and phosphorous metabolites as well as organic matter. Most of the microbial species flourishing within the aquaculture systems and their specific roles remain mystifying. In this regard, metagenomics can provide

additional information regarding the understanding of the microbial diversity that thrives within the aquaculture systems. The present study is a preliminary attempt to explore the microbial diversity present in the gut of an aquaculture pond employing metagenomics. Next-generation sequencing of the gut sample revealed distinct and diverse microbial communities present in the sample. 16s metagenomic analysis of the sample was performed in Phytocom Pharmaceuticals (P)LTD,Kalamassery,Kerala, India.Analysis of the results showed a high abundance of Betaproteobacteria in the metagenomeretrieved from the gut sample followed by Alphaproteobacteria,Clostridia,Actinobacteria,Gammaproteobacteria and Bacilli in the metagenome retrieved from the gut sample.Figure 1 shows the relative abundance of the most dominant bacterial groups.

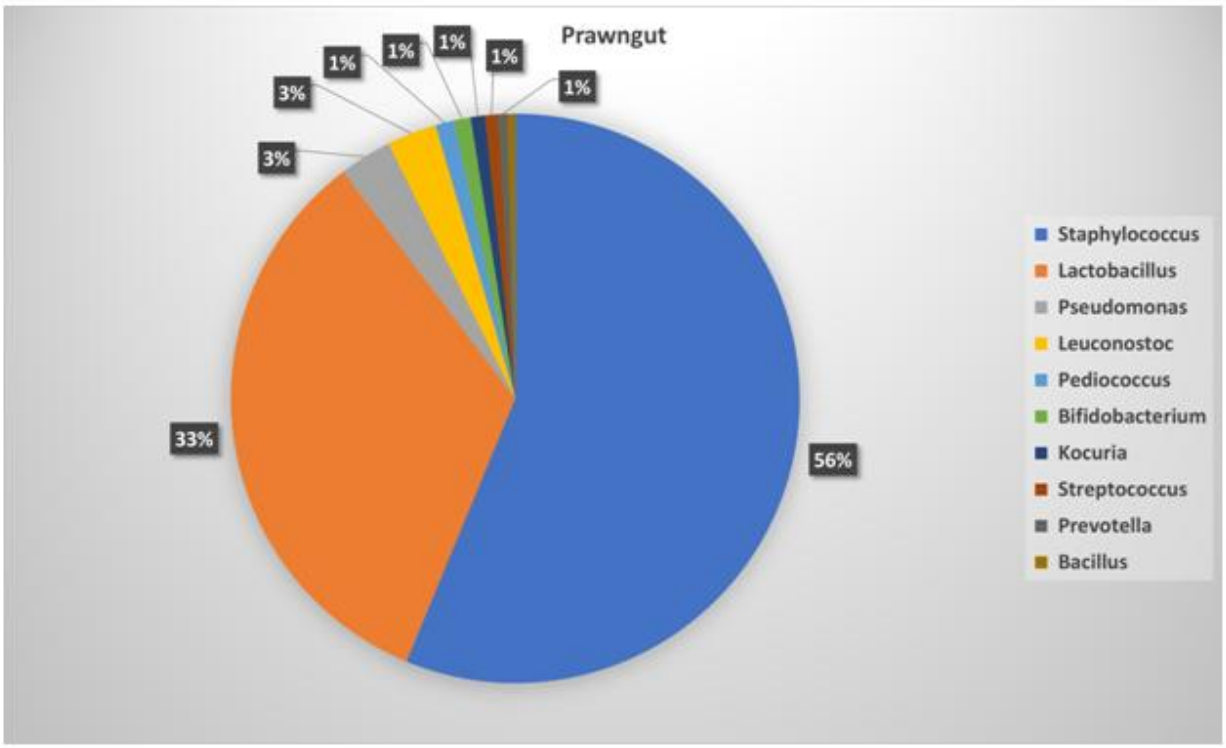

Fig 1: Top 10 genus of Shrimp gut

Betaproteobacteria was found to be the most abundant phylum in the metagenome retrieved from the gut sample. Betaproteobacteria and Alphaproteobacteria come under the class Proteobacteria. Proteobacteria play essential roles in nutrient cycling and mineralization of organic compounds and are found to be widely distributed in the marine environment. Previous studies have reported that proteobacteria dominate the gut microbiome of penaeid shrimps. Proteobacteria have been reported to be more abundant in the shrimp intestines and are associated with slow growth performance and potential risk of disease. Some of the bacteria from this phylum are responsible for nitrogen fixation also. Most of the OTUs

assigned to this phylum were assigned to be Vibrio harveyi, the potential shrimp pathogen that usually results in mass mortality. The high abundance of V. harveyi in the gut shows the risk associated with the pond. Another potential pathogen that could detected in the gut samples was Acinetobacter lwoffii which is also a proteobacterium. The next abundant phyla of the gut sample wasVerrucomicrobia, capable of oxidizing a range of complex polymeric carbon compounds, enhancing the capacity of organic matter degradation in toxic sediments. Bacteroidetes are a group of the intestinal microbiome that are beneficial to the host organism and abundant in the gut samples. This phylum includes some of the most abundant groups in the marine systems after proteobacteria. Most of the OTUs assigned to this phylum were further classified as belonging to the class Flavobacteria and the order Flavobacteriales. Flavobacteria are considered potential bioremediators of the culture systems and play an important role in the degradation of organic matter. Species of the genus Bacteroidetes have been reported to show high antibiotic resistance capacity and have been reported as major vitamin B12 producers in the intestines of shrimps and finfishes.Other abundant phyla identified in the gut samples were beneficial bacteria belonging to Clostridia.Clostridia are strictly anaerobic to aerotolerant sporeforming bacilli found in soil as well as in normal intestinal flora of man and animals.

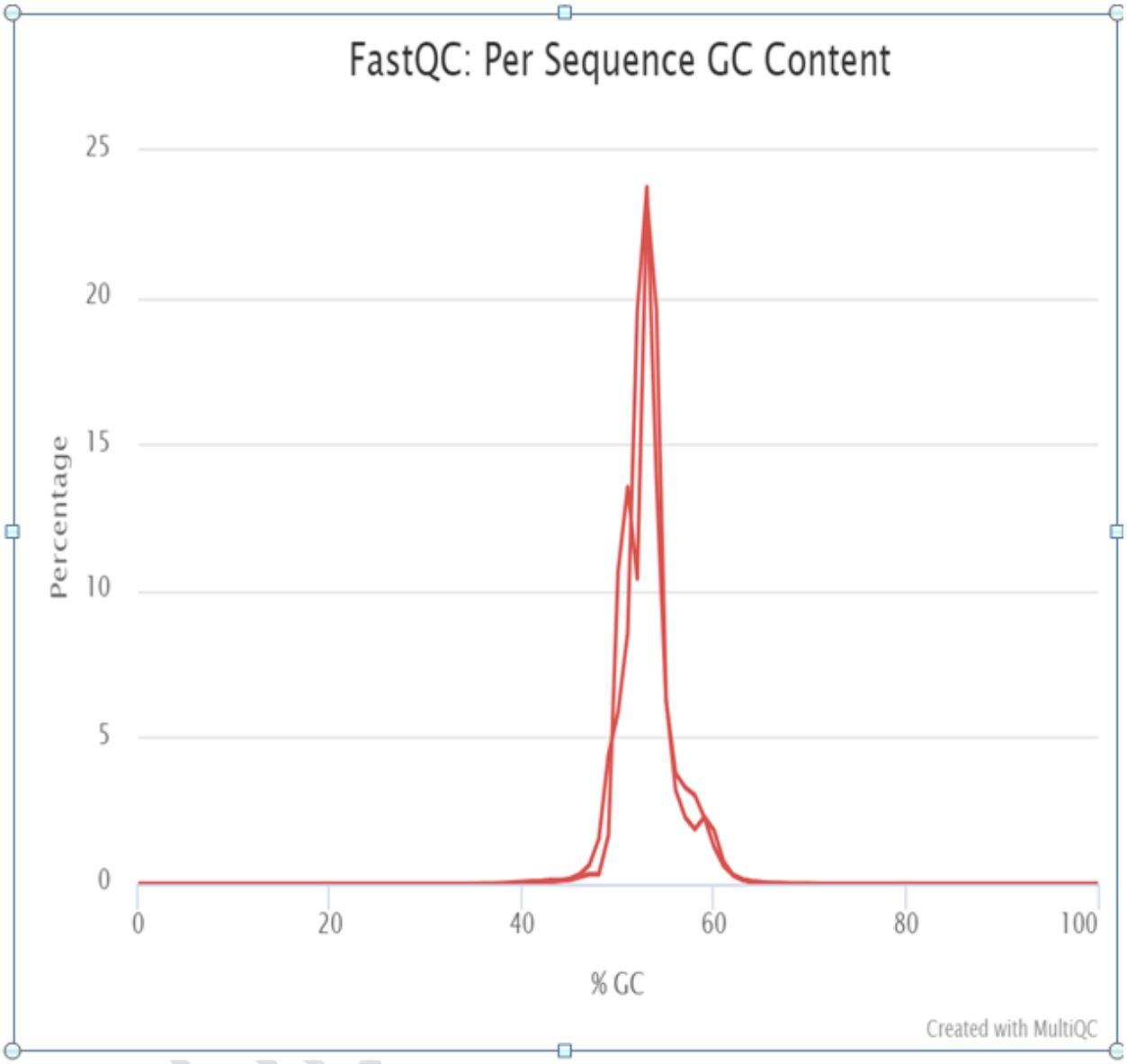

Fig 2: Quality control matrices of sequences

Operational taxonomic units (OTUs) cluster analysis showed that the two samples tested were divided into 24 phyla and 230 genera.Percentile calculation of phylum,class,order,family,genus, and speciesof shrimp gut observed from metagenomic analysis were obtained as follows.Firmicutes(90-95%),Proteobacteria(85-90%),Actinobacteria(80-85%)Bacteriodetesand Fusobacteria (75-80%) were the most abundant phyla. At the class level, Bacilli (90-95%), was the most abundant class. Gammaproteobacteria (85-90%), was the second most abundant class.Actinobacteria(80-85%),Clostridia(75-80%)),Alphaproteobacteria and Betaproteobacteria were the topmost classes identified by OUT clustering analysis.Bacillales(90-95%),Lactobacillales(85-90%) and Pseudomonales(80- 85%),Micrococcales,Enterobacterales,Clostridiales (75-80)were the top most abundant orders . When the OTUs were considered at the genus level, a high diversity of microbes was identified. A total of 230 genera were detected in all the samples. The genus level accounting for the largest proportion was Staphylococcus (90-95%).Thetop dominant genera were Staphylococcus,Lactobacillus,Pseudomonas,Leuconostoc,Pediococcus,Bifidobacterium,Kocuria,Streptococcus,Prevotella,Bacillus,Flavobacterium.A total of 230 genera were identified from shrimp gut OUT clustering analysis.162 families and 85 Orders were identified.46 classes and 24 phyla were identified.Detailed

illustrations of the shrimp gut microbiotacommunities are explained in the following figures.

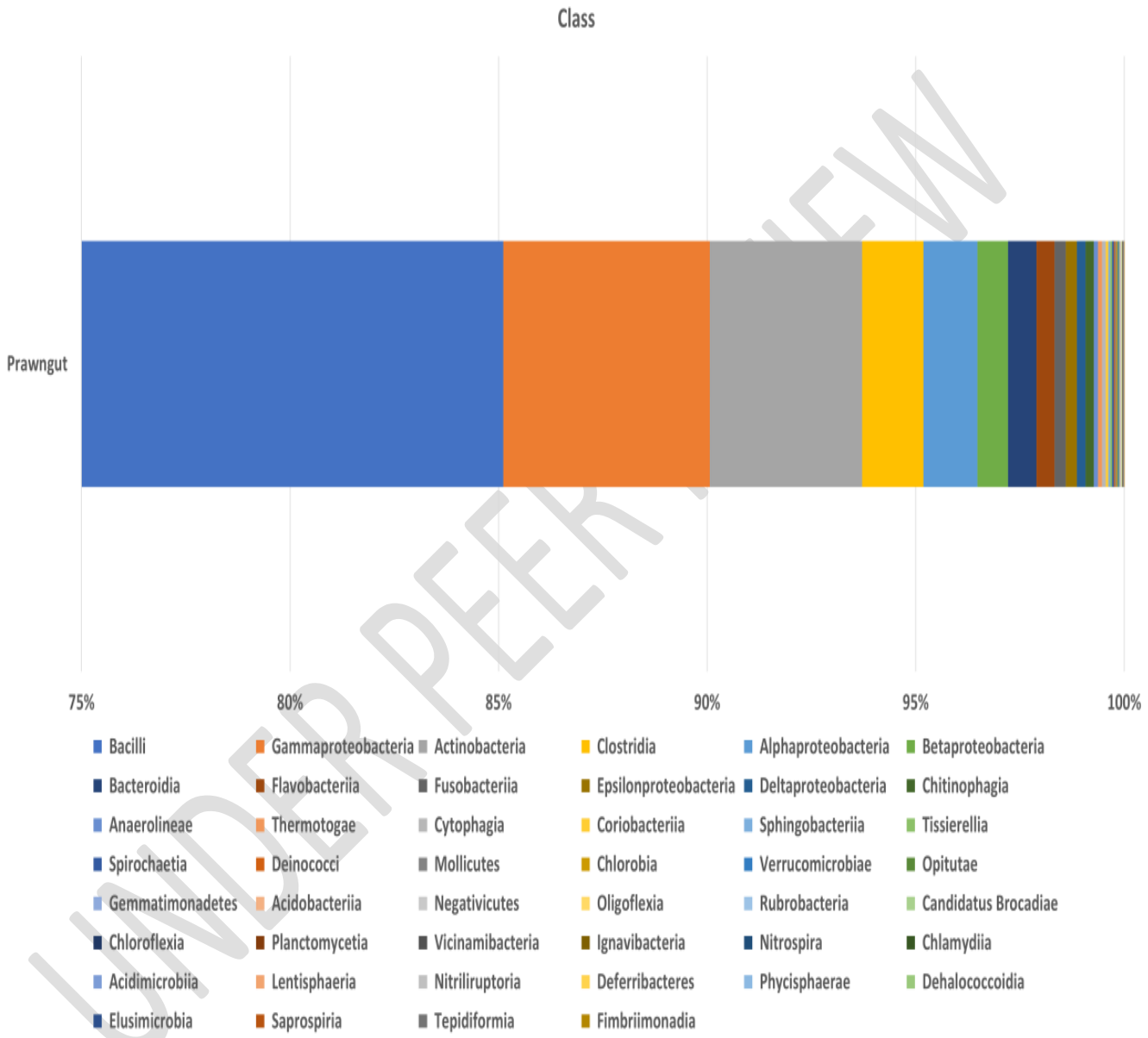

Fig 3 : Analysed classes of *Penaeus monodon*

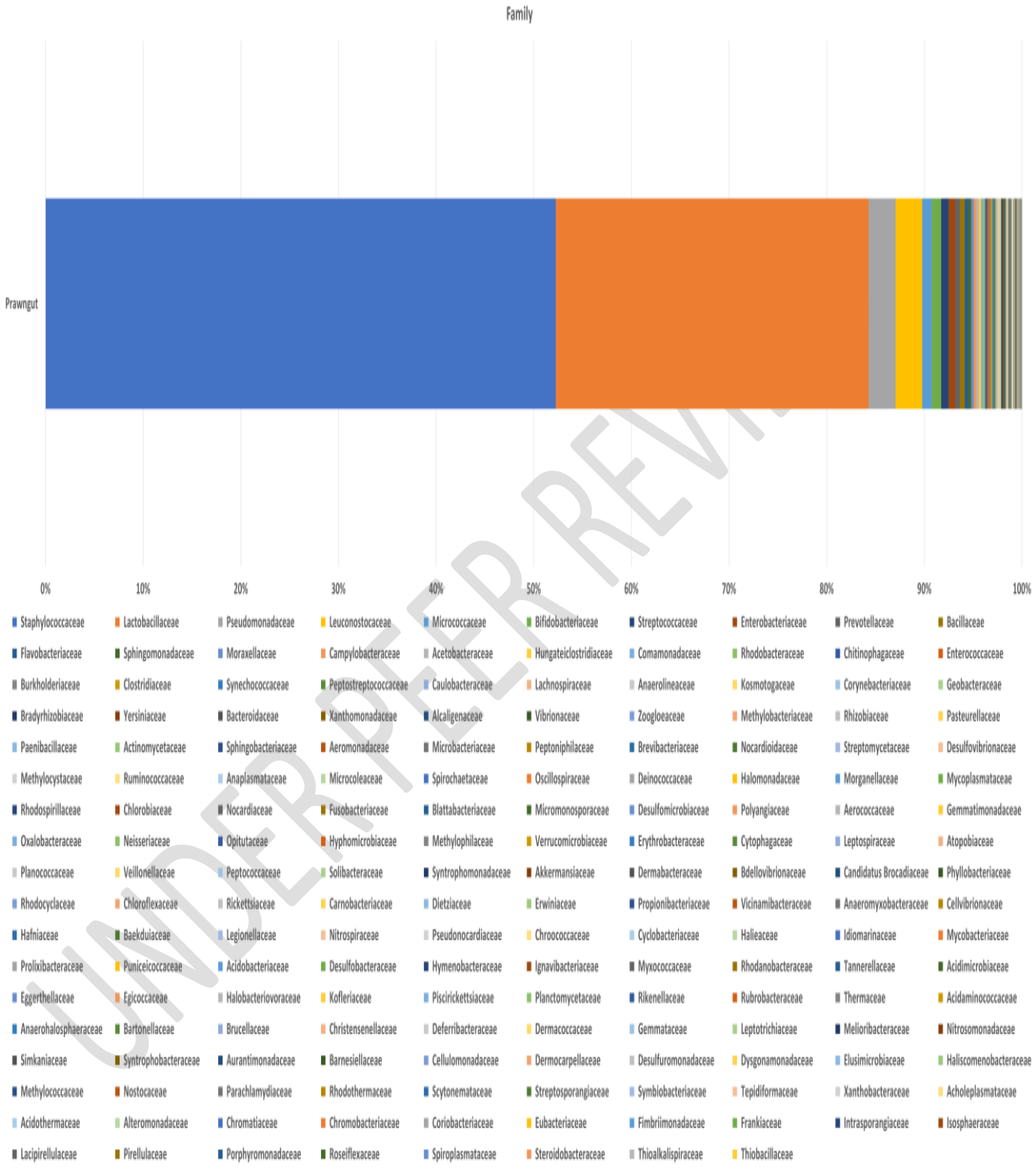

Fig 4 : Analysed families of *Penaeus monodon*

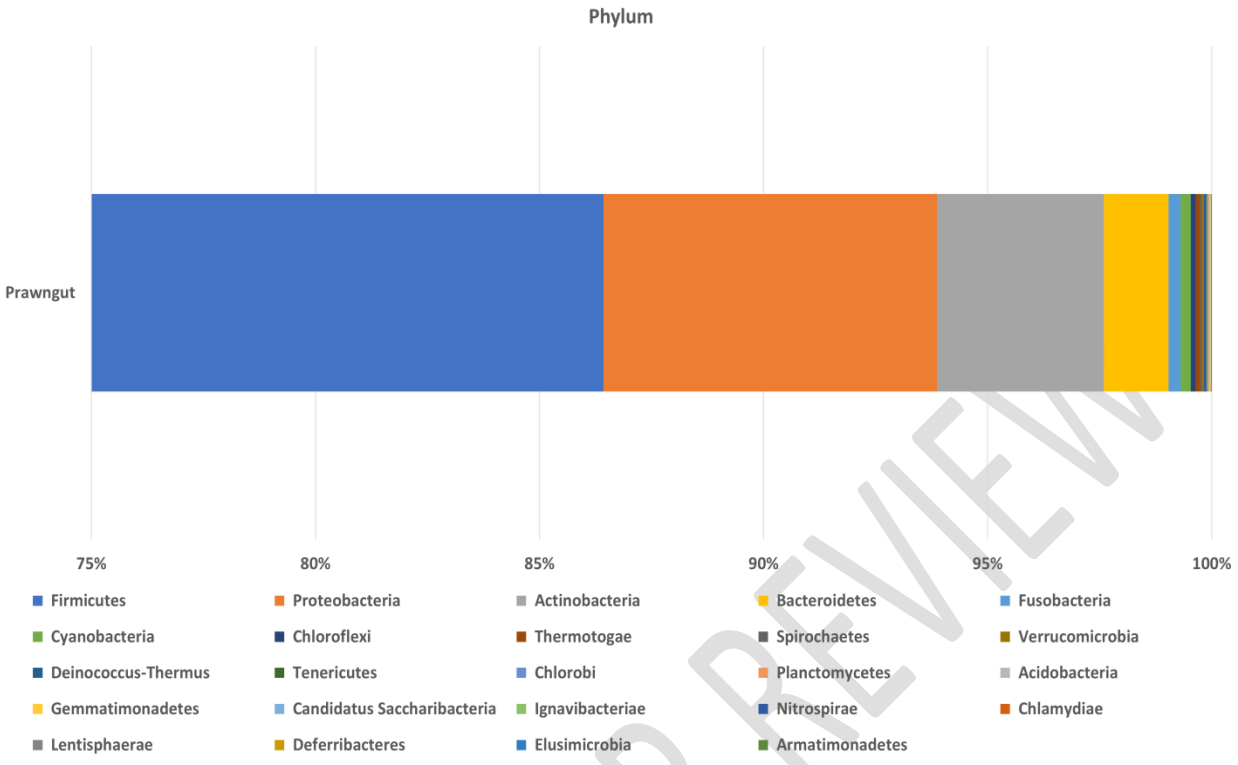

Fig 5: Analysed phylum of *Penaeus monodon*

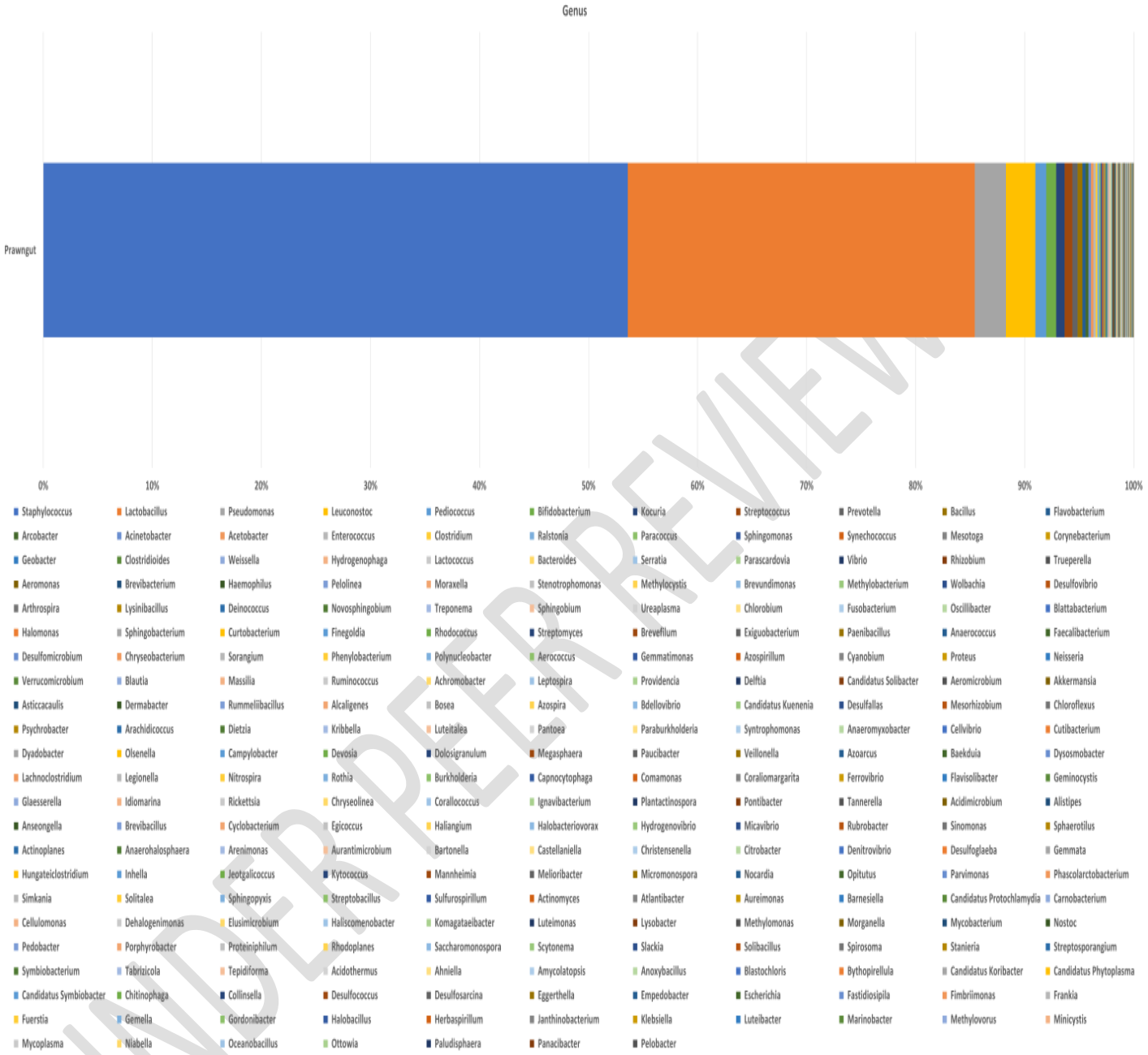

Fig 6: Analysed genus of *Penaeus monodon*

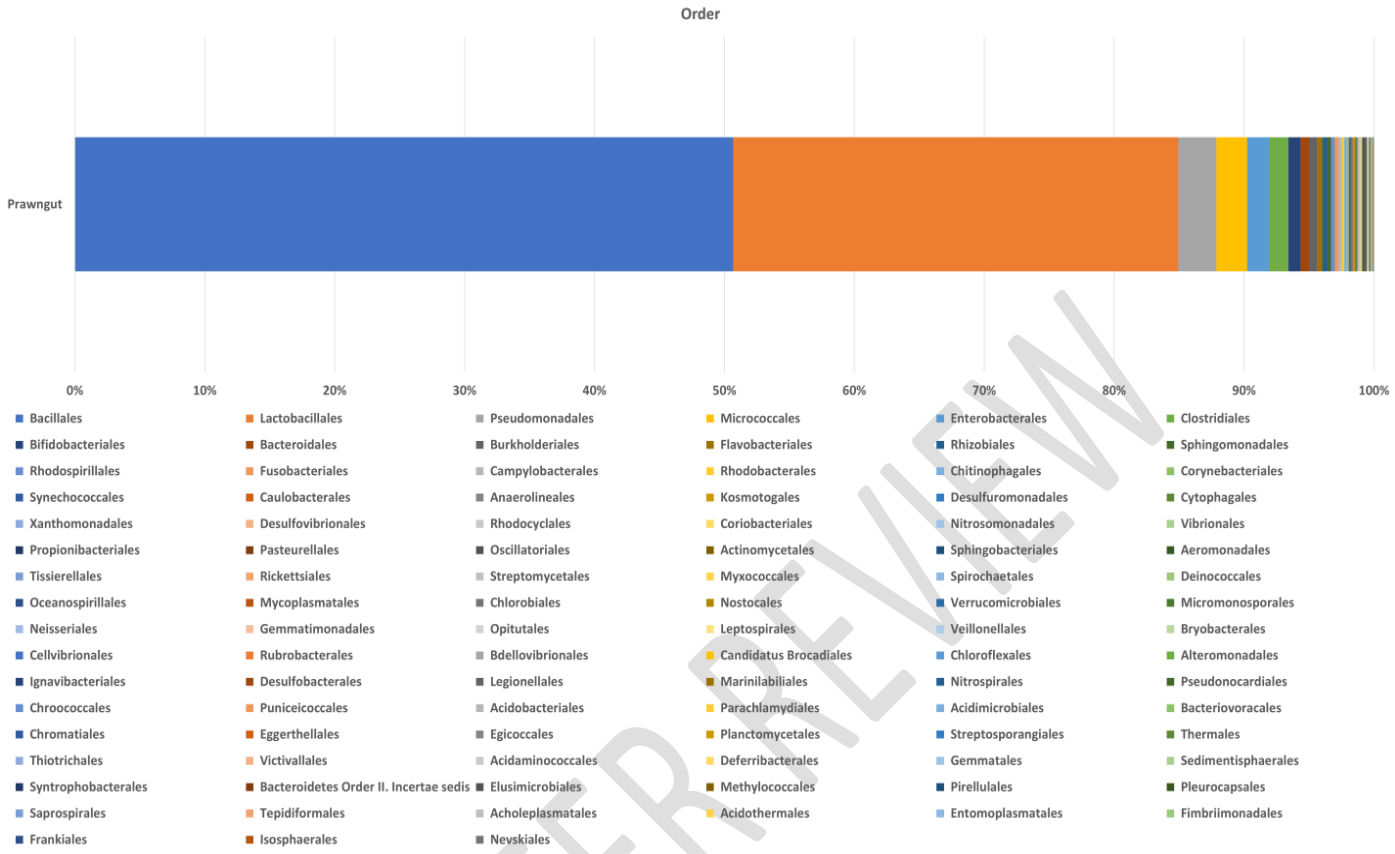

Fig 7: Analysed order of *Penaeus monodon*

DISCUSSION AND CONCLUSION

Metagenomic analysis of the aquaculture systems will pavethe way for elucidating the diversity of microorganisms present in the system and their potential role in the aquaculture system, including the determination of metabolic processes performed by microbes; understanding the biogeochemical cycles of nutrients in the culture systems as well the development/outbreak of diseases. In conclusion, taxonomic profiles of microbiotas in the sediment of shrimp farming environments were investigated in this study employing metagenomics. The present study provides preliminary data concerning the microbial community present in the gut of a semi-intensive shrimp culture system. Microbes are the most dominant group that harbours much in the sediments of shrimp ponds. The metagenomic analysis provides a better idea about the microbial communities present in an aquaculture system, especially the uncultivable ones. The present study emphasizes the application of metagenomics in exploring the microbial diversity of aquaculture systems, which might help detect pathogens within the system and help to develop pathogen control strategies in the aquaculture systems.

Disclaimer

This paper is an extended version of a preprint document of the same author.

The preprint document is available in this link: https://www.researchsquare.com/article/rs-3222901/v1

[As per journal policy, preprint article can be published as a journal article, provided it is not published in any other journal]

References

JPEN J Parenter Enteral Nutr 23, S70–S73. Bacanu, G.M. and Oprea, L. (2013) Differences in the gut microbiota between wild and domestic Acipenser ruthenus evaluated by denaturing gradient gel electrophoresis. Rom Biotechnol Lett 18, 8069–8076.

Balcazar, J.L., de Blas, I., Ruiz-Zarzuela, I., Cunningham, D., Vendrell, D. and Muzquiz, J.L. (2006) The role of probiotics in aquaculture. Vet Microbiol 114, 173–186.

Bennett, K.W. and Eley, A. (1993) Fusobacteria – new taxonomy and related diseases. J Med Microbiol 39, 246–254. Bourgault, A.M., Lamothe,

F., Dolce, P., SaintJean, L. and SaintAntoine, P. (1997) Fusobacterium bacteremia: clinical experience with 40 cases.

Clin Infect Dis 25, S181–S183. Cipriano, R.C., Bullock, G.L. and Pyle, S.W. (1984) Aeromonas hydrophila and Motile Aeromonad Septicemias of Fish. US Department of the Interior, US Fish and Wildlife Service, Divison of Fishery Research, Fish Disease Leaflet, 68, Washington.

Clarke, K.R. and Gorley, R.N. (2006) PRIMER v6: User Manual/Tutorial. Plymouth, UK: Primer-e. Clements, K.D. and Choat, J.H. (1995) Fermentation in tropical marine herbivorous fishes. Physiol Zool 68, 355–378.

Clements, K.D., Gleeson, V.P. and Slaytor, M. (1994) Shortchain fatty-acid metabolism in temperate marine herbivorous fish. J Comp Physiol B 164, 372–377.

Collinder, E., Bjornhag, G., Cardona, M., Norin, E., Rehbinder, C. and Midtvedt, T. (2003) Gastrointestinal host-microbial interactions in mammals

and fish: comparative studies in man, mice, rats, pigs, horses, cows, elk, reindeer, salmon and cod. Microb Ecol Health Dis 15, 66–78.

DeSantis, T.Z., Hugenholtz, P., Larsen, N., Rojas, M., Brodie, E.L., Keller, K., Huber, T., Dalevi, D. et al. (2006) Greengenes, a chimera-checked 16S rRNA gene database and workbench compatible with ARB. Appl Environ Microbiol 72, 5069–5072.

Di Maiuta, N., Schwarzentruber, P., Schenker, M. and Schoelkopf, J. (2013) Microbial population dynamics in the faeces of wood-eating loricariid catfishes. Lett Appl Microbiol 56, 401–407.

Donkeng, N.N., Maiwore, J., Ngoune, L.T., Montet, D. and Mbofung, C.M.F. (2011) Characterization of the bacterial flora of tilapia (Oreochoromis niloticus) harvested from four lakes in the north of Cameroon.

Afr J Biotechnol 10, 16016–16023. Von Engelhardt, W., Bartels, J., Kirschberger, S., Duttingdorf, H.D.M.Z. and Busche, R. (1998) Role of short-chain fatty acids in the hind gut. Vet Q 20, S52–S59.

Azim ME, Little DC (2008) The biofloc technology (BFT) in indoor tanks: water quality, biofloc composition, and growth and welfare of Nile tilapia (Oreochromis niloticus).

Glencross BD, Booth M, Allan GL (2007) A feed is only as good as its ingredients–a review of ingredient evaluation strategies for aquaculture feeds. Aquac Nutr 13(1):17–34. https://doi.org/10.1111/j.1365-2095.2007.004 50.x

Troell M, Joyce A, Chopin T, Neori A, Buschmann AH, Fang JG (2009) Ecological engineering in aquaculture-potential for integrated multi-trophic aquaculture (IMTA) in marine offshore systems. Aquaculture 297(1-4):1–9. https://doi.org/10.1016/j.aquaculture.2009.09.010

Caruso G (2013) Microbes and their use as indicators of pollution. J Pollut Eff Cont 1:e102. https://doi.org/10.4172/jpe.1000e102 .

FDA (2011) Aquaculture Drugs. In Fish and Fishery Products Hazards and Controls Guide ed. U. S. Department of Health and Human Services pp.

183–207. Washington, DC: FDA, Center for Food Safety and Applied Nutition, Office of Food Safety.

FDA (2012) Animal Husbandry and Disease Control: Aquaculture. Silver Spring, MD: U. S. Food and Drug Administration.

Finegold, S.M., Vaisanen, M.L., Molitoris, D.R., Tomzynski, T.J., Song, Y., Liu, C., Collins, M.D. and Lawson, P.A. (2003) Cetobacteriumsomerae sp. nov from human feces and emended description of the genus Cetobacterium. SystApplMicrobiol 26, 177–181.

Gao, Y.L., Storebakken, T., Shearer, K.D., Penn, M. and Overland, M. (2011) Supplementation of fishmeal and plant protein-based diets for rainbow trout with a mixture of sodium formate and butyrate.

Aquaculture 311, 233–240. Griswold, B.L. and Tubb, R.A. (1977) Food of yellow perch, white bass, freshwater drum, and channel catfish in Sandusky Bay, Lake Erie.

Ohio J Sci 77, 43–47. Hamer, H., Jonkers, D., Troost, F.J., Bast, A., Vanhoutvin, S., Venema, K. and Brummer, R.J. (2007) Butyrate modulates oxidative stress in the colonic mucosa of healthy humans. Gastroenterology 132, A79.

Hargreaves, J.E. and Lucey, D.R. (1990) Life-threatening Edwardsiellatarda soft-tissue infection associated with catfish puncture wound. J Infect Dis 162, 1416–1417.

Harris, N.J. (1999) Seasonal food habits of bluegills in Richmond Lake, South Dakota. Proc S Dak Acad Sci 78, 79–85.

Heikkinen, J., Vielma, J., Kemilainen, O., Tiirola, M., Eskelinen, P., Kiuru, T., Navia-Paldanius, D. and von Wright, A. (2006) Effects of soybean meal based diet on growth performance, gut histopathology and intestinal microbiota of juvenile rainbow trout (Oncorhynchus mykiss).