



Full Length Article

Illumina Sequencing Analysis of the Microbial Community in the Gastrointestinal Tract of Chinese Mitten Crab (*Eriocheir sinensis*)

Yanfeng Zhou¹, Yanfen Cheng², Bei Jin¹ and Yang You^{1*}

¹Freshwater Fisheries Research Center, Chinese Academy of Fishery Science, Wuxi, 214081, China

²College of Animal Science and Technology, Nanjing Agricultural University, Nanjing, 210095, China

*For correspondence: youy@ffrc.cn

Abstract

The Chinese mitten crab (big sluice crab, *Eriocheir sinensis*) is one of the most economically important aquatic species in China. The present study was aimed to investigate the microbial community and their potential functions in the gastrointestinal (GI) tract of the crab. The whole gut was collected for DNA extraction and subsequent Illumina MiSeq sequencing of the bacterial 16S rRNA gene. A total of 786,410 valid sequences and 752 operational taxonomic units (OTUs) were obtained. The number of OTUs observed in the GI tract of the crab was 190.04 ± 5.59 and the Shannon index was 4.31 ± 0.12 . The dominant phyla included Tenericutes, Bacteroidetes, Proteobacteria and Firmicutes. The predominant genera were unassigned and supposedly belong to the class of Mollicutes, which accounted for 41.64% in average. The dominant genera also included five assigned genera, which were *Bacteroides*, *Vibrio*, *Shewanella*, *Candidatus Hepatoplasma*, *Dysgonomonas* and four unassigned genera, which belong to the classes of Mollicutes, Alphaproteobacteria, Bacteroidia, and Gammaproteobacteria. No significant difference was observed in microbial community between male and female crabs. Functional prediction with PICRUSt suggested that the microbes in the GI tract of the crab were mainly involved in replication and repair, carbohydrate and amino acid metabolism, membrane transport, and translation. The microbes in the GI tract of the crab may play key roles in the metabolism of nutrients. Further studies are needed to control the microbial community to improve the feed efficiency of the Chinese mitten crab. © 2019 Friends Science Publishers

Keywords: Chinese mitten crab; Microbial community; Miseq; PICRUSt; 16S rRNA gene

Introduction

The microbial community in the GI tract plays an important role in nutrient metabolism and absorption and in innate immunity (Rawls *et al.*, 2004). Its composition and function have been well studied in many organisms, including humans, pigs, ruminants, etc. Lewis *et al.* (2017) reported that *Bifidobacterium* was the predominant bacterial genus in the feces of infants. Hsieh *et al.* (2016) reported that Firmicutes and Bacteroidetes were the dominant bacterial phyla in human feces. Ji *et al.* (2017) reported that Firmicutes and Proteobacteria were the dominant phyla in the ileal contents of pregnant Huanjiang mini-pigs. Liu *et al.* (2016) reported that Firmicutes, Bacteroidetes, Fibrobacteres, Spirochaetae and Tenericutes were the dominant phyla in the rumen of cows. However, only limited reports have focused on the microbial community and its function in the GI tract of aquatic organisms, including fish, shrimps and crabs. Li *et al.* (2016) reported that the dominant phyla were Actinobacteria, Bacteroidetes, Firmicutes, Fusobacteria, Proteobacteria, Spirochaetes and Tenericutes in the GI tract of wild largemouth bronze gudgeon. Ghanbari *et al.* (2016) investigated the intestinal

microbes of snow trout and identified the dominant phyla as Firmicutes, Proteobacteria, Fusobacteria, Actinobacteria, Planctomycetes, Bacteroidetes, Cyanobacteria and Tenericutes. van Kessel *et al.* (2011) found that the dominant phyla were Proteobacteria, Firmicutes, Bacteroidetes, Fusobacteria, Planctomycetes and Verrucomicrobia in the GI tract of carp (*Cyprinus carpio* L.). Cardona *et al.* (2016) demonstrated that the dominant phyla were Proteobacteria, Firmicutes, Bacteroidetes and Tenericutes in the GI tract of shrimp. Meziti *et al.* (2012) reported that the predominant phyla in the GI tract of reared *Nephrops norvegicus* were Proteobacteria.

The Chinese mitten crab (*Eriocheir sinensis*) is one of the most economically important aquatic species in China (24.5 million US dollars in 2015). The dominant bacteria in the GI tract of this crab are reported as Proteobacteria, Bacteroidetes, Actinobacteria, and Verrucomicrobia by denaturing gradient gel electrophoresis and a 16S rRNA gene clone library (Li *et al.*, 2007). However, Di *et al.* (2014) reported that the dominant bacteria in the GI tract of this crab were Proteobacteria, Firmicutes, and Bacteroidetes by culture-dependent methods. One solution to this discrepancy could be the use of next generation

sequencing, a powerful new technique (Wang *et al.*, 2018; Yang *et al.*, 2019), to investigate the microbial diversity in the GI tract of this crab. The aim of the present study was therefore to apply the 16S rRNA gene Illumina sequencing approach to investigate the microbial community and its potential functions in the GI tract of male and female Chinese mitten crabs in order to improve the feed efficiency, health and meat quality of this crab.

Materials and Methods

Sample Collection

In August 2015, 24 crabs were collected (each in duplicate) in Suzhou and Wuxi, Jiangsu Province in China. The temperature, pH and dissolved oxygen of the water are $31.2 \pm 0.3^\circ\text{C}$, 8.19 ± 0.13 and 7.14 ± 0.15 mg/L, respectively. The feed contains >40% crude protein, >4% ether extract, <8% crude fiber, <15% ash and <12.5% water. The concentrations of total phosphorus and lysine in the feed are >1% and >1.7%, respectively. The whole guts from two crabs were combined to obtain enough samples for DNA extraction. The detailed information on 48 crabs is shown in Table 1.

DNA Extraction and MiSeq Sequencing

The metagenomic DNA was extracted with a marine animal DNA kit (Tiangen, Beijing, China) according to the manufacturer's protocol. The extracted DNA was checked with 1% agarose gel and stored at -20°C .

The universal primers (338F 5'-ACTCCTACGGGAGGCAGCAG-3' and 806R 5'-GGACTACHVGGGTWTCTAAT-3'), targeting the V3–V4 regions of the bacterial 16S rRNA gene, were used for PCR amplification (Behrendt *et al.*, 2012). The amplicons were then paired-end sequenced on an Illumina MiSeq platform by Majorbio (Shanghai, China). The raw reads were deposited in SRA under the accession number: SRP110220.

Bioinformatics Analysis

The paired-end reads for each sample were joined with QIIME (Caporaso *et al.*, 2010). The sequences were then quality filtered to exclude all sequences <200 or >500 bp, with ambiguous bases, and with a mean quality score of <30. The filtered sequences were then used for operational taxonomic units (OTUs) picking at the level of 97% with QIIME (Greengenes database as reference). The taxonomic assignment of the representative sequence of each OTU was also analyzed in QIIME, and the alpha diversity was calculated at the depth of 17,050 sequences. The KEGG classified functions of the microbes in the GI tract of the crab were predicted by PICRUSt (Langille *et al.*, 2013) from the website: <http://huttenhower.sph.harvard.edu/galaxy/>.

Statistical Analysis

The relative abundances of microbes were presented as mean \pm SEM. The microbial difference between male and female crabs was analyzed using ANOVA with the software SPSS 19.0 (IBM, USA). Significant differences were declared at $P < 0.05$. Data were visualized with ggplot2 in R (Chang, 2012).

Results

Alpha Diversity

A total of 786,410 valid sequences were obtained, with an average of 32,767 sequences per sample. The number of 752 OTUs was picked at the minimum sequence similarity of 97%. The Good's coverage of the samples ranged from 99.57 to 99.91%. The Observed_otus and Shannon indexes were 190.04 ± 5.59 and 4.31 ± 0.12 , respectively.

The Microbial Community in the GI Tract of Chinese Mitten Crab

The relative abundances of five phyla, including Bacteroidetes, Firmicutes, Proteobacteria, Tenericutes and TM7, were above 1% in at least one sample (Fig. 1A). The average relative abundances of phyla showed that Tenericutes was the predominant phylum in the GI tract of the crab, with an average relative abundance of $57.70 \pm 4.33\%$, followed by Bacteroidetes ($17.44 \pm 2.69\%$), Proteobacteria ($16.75 \pm 1.98\%$) and Firmicutes ($7.45 \pm 0.88\%$; Fig. 1B).

The predominant order was unclassified, which belonged to the class of Mollicutes, with the relative abundance of $41.64 \pm 4.24\%$. It was followed by an unclassified order belonging to the class of Alphaproteobacteria ($6.42 \pm 1.26\%$), Clostridiales ($3.93 \pm 0.58\%$), Vibrionales ($3.78 \pm 1.23\%$), Alteromonadales ($2.49 \pm 0.60\%$), Bacillales ($1.75 \pm 0.37\%$), Enterobacteriales ($1.62 \pm 0.39\%$), Erysipelotrichales ($1.23 \pm 0.27\%$) and Pseudomonadales ($1.11 \pm 0.44\%$; Fig. 2).

The predominant genus was unclassified, which belonged to the class of Mollicutes, with a relative abundance of $41.64 \pm 4.24\%$ (Fig. 3). At the OTU level, the average relative abundances of 21 OTUs were above 1%. The predominant OTU, belonging to the order of Bacteroidales, accounted for $7.32 \pm 1.79\%$ (Fig. 4).

Functional Prediction of Microbes in the GI Tract of the Chinese Mitten Crab

The predicted function of the microbes in the GI tract of the Chinese mitten crab is given in Fig. 5. The number of 328, 39 and 8 KEGG functions was predicted at levels 3, 2 and 1, respectively. Within 39 predicted functions at level 2, the predominant one was replication and repair (Genetic Information Processing), followed by carbohydrate

Table 1: Details of the samples analyzed in this study

Sample ID	Width	Length	Hight	Weight	Sex	City	Location
Crab1	57.74	57.27	32.12	102.39	Male	Suzhou	Yangcheng lake
	60.20	56.31	29.32	103.80	Male	Suzhou	Yangcheng lake
Crab2	60.79	57.68	31.09	104.15	Male	Suzhou	Yangcheng lake
	60.37	56.77	29.78	108.19	Male	Suzhou	Yangcheng lake
Crab3	58.94	56.81	31.06	109.60	Male	Suzhou	Yangcheng lake
	60.41	56.77	29.47	103.91	Male	Suzhou	Yangcheng lake
Crab4	61.73	58.50	33.12	110.60	Female	Suzhou	Yangcheng lake
	60.80	56.39	32.84	106.35	Female	Suzhou	Yangcheng lake
Crab5	58.03	56.28	31.02	101.51	Female	Suzhou	Yangcheng lake
	60.69	56.31	30.67	101.22	Female	Suzhou	Yangcheng lake
Crab6	58.34	55.77	31.36	103.61	Female	Suzhou	Yangcheng lake
	60.69	56.31	30.67	100.45	Female	Suzhou	Yangcheng lake
Crab7	52.40	49.66	26.40	71.32	Male	Suzhou	Yangcheng lake
	52.46	49.35	26.08	71.70	Male	Suzhou	Yangcheng lake
Crab8	54.33	50.16	26.53	72.08	Male	Suzhou	Yangcheng lake
	53.96	51.30	27.90	72.22	Male	Suzhou	Yangcheng lake
Crab9	52.36	50.25	27.47	72.93	Male	Suzhou	Yangcheng lake
	55.57	50.31	26.30	74.02	Male	Suzhou	Yangcheng lake
Crab10	58.19	53.41	28.17	93.76	Male	Suzhou	Yangcheng lake
	58.39	55.29	30.49	91.07	Male	Suzhou	Yangcheng lake
Crab11	54.73	52.50	27.82	92.02	Male	Suzhou	Yangcheng lake
	56.06	54.41	29.03	92.47	Male	Suzhou	Yangcheng lake
Crab12	56.80	54.39	28.84	92.72	Male	Suzhou	Yangcheng lake
	56.27	56.24	31.07	93.79	Male	Suzhou	Yangcheng lake
Crab13	61.56	52.45	30.23	95.20	Male	Suzhou	Yangcheng lake
	58.85	56.32	30.88	96.05	Male	Suzhou	Yangcheng lake
Crab14	59.70	56.70	30.70	97.50	Male	Suzhou	Yangcheng lake
	57.80	56.30	30.89	98.43	Male	Suzhou	Yangcheng lake
Crab15	58.14	56.62	30.20	99.22	Male	Suzhou	Yangcheng lake
	61.40	53.49	30.22	96.60	Male	Suzhou	Yangcheng lake
Crab16	57.61	54.06	26.92	90.14	Male	Suzhou	Pond1
	57.97	53.63	28.63	90.40	Male	Suzhou	Pond1
Crab17	57.12	54.03	27.16	90.57	Male	Suzhou	Pond1
	57.67	52.87	27.41	90.69	Male	Suzhou	Pond1
Crab18	54.47	49.16	24.93	92.45	Male	Suzhou	Pond1
	58.75	55.74	30.10	92.81	Male	Suzhou	Pond1
Crab19	54.95	52.2	27.35	77.64	Female	Wuxi	Pond2
	50.77	45.38	22.58	48.84	Female	Wuxi	Pond2
Crab20	59.2	55.8	29.84	86.37	Female	Wuxi	Pond2
	57.72	48.9	27.58	77.56	Female	Wuxi	Pond2
Crab21	57.57	51.16	27.53	87.18	Female	Wuxi	Pond2
	54.91	50.6	24.78	64.96	Female	Wuxi	Pond2
Crab22	57.29	49.98	26.16	72.18	Female	Wuxi	Pond3
	51.15	47.67	23.29	66.13	Female	Wuxi	Pond3
Crab23	48.15	44.72	22.6	47.34	Female	Wuxi	Pond3
	53.17	49.13	25.58	72.04	Female	Wuxi	Pond3
Crab24	54.07	48.69	24.6	60.71	Female	Wuxi	Pond3
	56.62	52.33	26.94	93.23	Female	Wuxi	Pond3

metabolism (Metabolism), amino acid metabolism (Metabolism), membrane transport (Environmental Information Processing) and translation (Genetic Information Processing). Interestingly, one function of cardiovascular diseases (Human Diseases) was predicted, although its relative abundance was low (0.01%).

Comparison of the Microbial Community in the GI Tract of Female and Male Chinese Mitten Crabs

The microbial community in the GI tract of female and male crabs was compared at the OTUs level. Only one of the 21 OTUs, with average relative abundances above 1%, had the tendency to increase in male crabs ($1.58 \pm 0.40\%$ vs $0.64 \pm 0.18\%$; $P=0.096$). No significant difference was observed among the other OTUs.

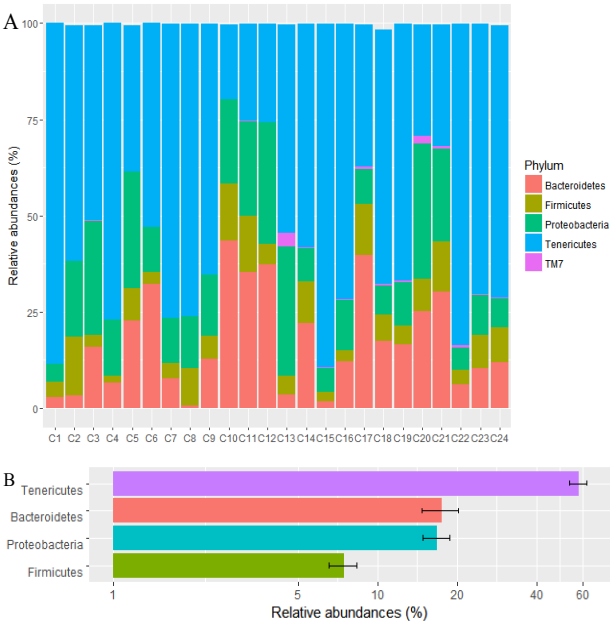


Fig. 1: Relative abundances (%) of bacterial phyla in the gastrointestinal tract of the crab (a, individual level of phyla above 1% in at least one sample; b, the phyla with the average relative abundances above 1%)

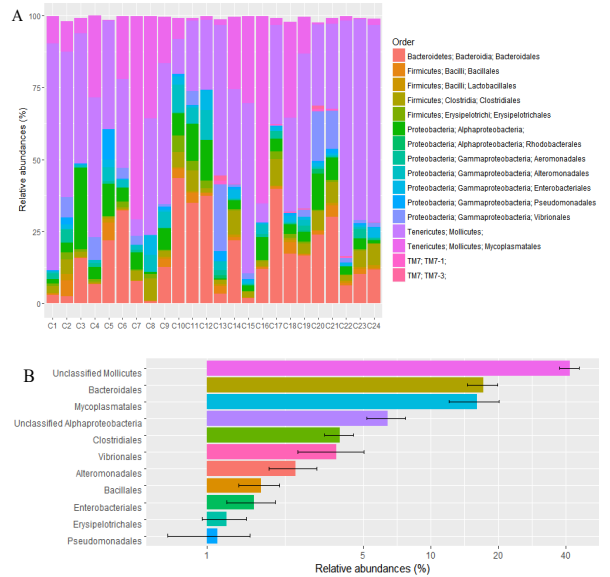


Fig. 2: Relative abundances (%) of bacterial orders in the gastrointestinal tract of the crab (a, individual level of orders above 1% in at least one sample; b, the orders with the average relative abundances above 1%)

Discussion

In the present study, Bacteroidetes, Firmicutes, Proteobacteria and Tenericutes were detected as the dominant phyla in the GI tract of the Chinese mitten crab. However, Li *et al.* (2007) reported that Proteobacteria,

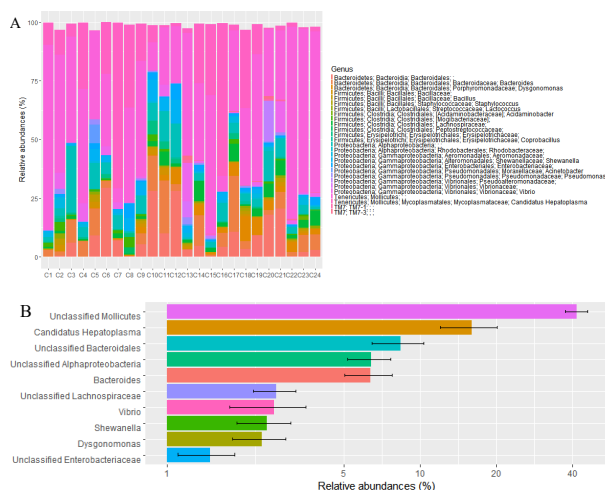


Fig. 3: Relative abundances (%) of bacterial genera in the gastrointestinal tract of the crab (a, individual level of genera above 1% in at least one sample; b, the genera with the average relative abundances above 1%)

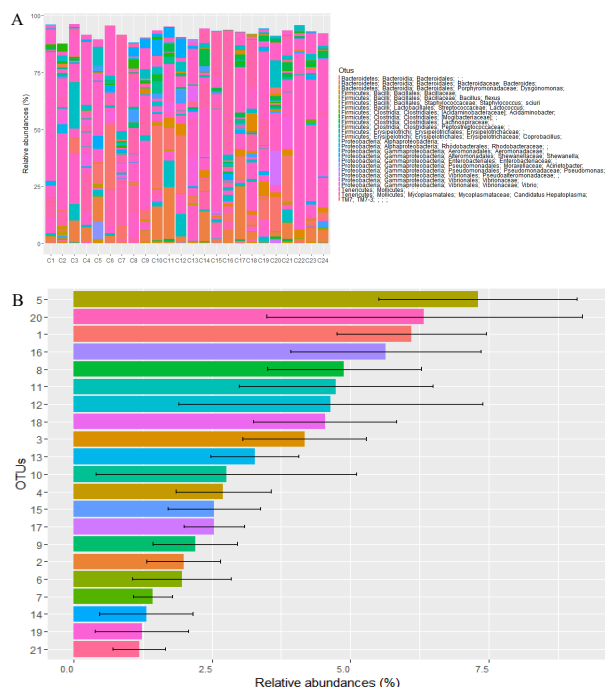


Fig. 4: Relative abundances (%) of bacterial OTUs in the gastrointestinal tract of the crab (a, individual level of OTUs above 1% in at least one sample; b, the OTUs with the average relative abundances above 1%)

Bacteroidetes, Actinobacteria, and Verrucomicrobia were the dominant phyla in the GI tract of this crab, as determined by denaturing gradient gel electrophoresis and a 16S rRNA gene clone library, whereas Di *et al.* (2014) reported that the dominant bacteria were Proteobacteria, Firmicutes and Bacteroidetes, as determined by a culture-dependent method. Yang *et al.* (2019) reported

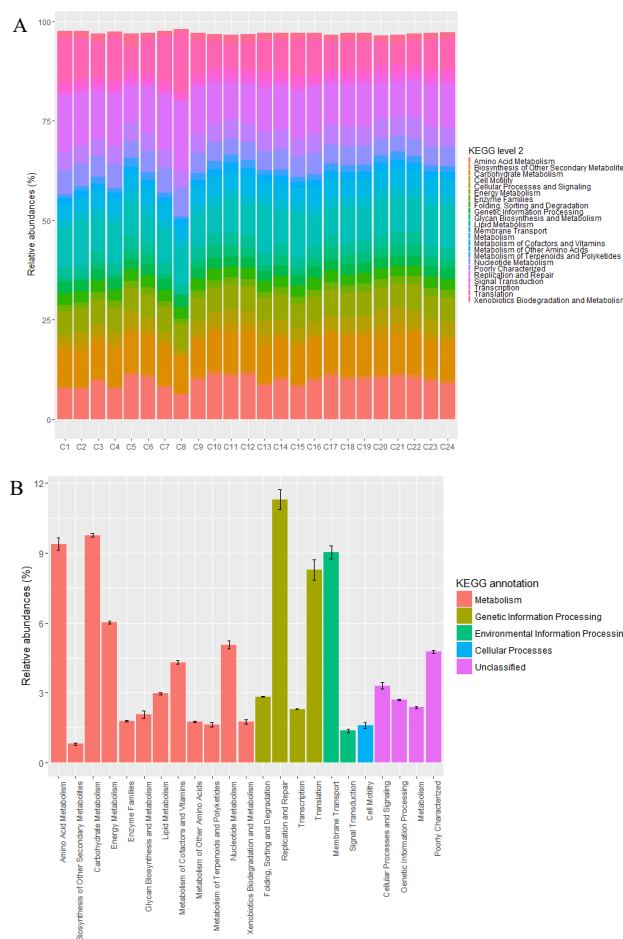


Fig. 5: The predicted function (relative abundance > 1% in at least one samples) of the microbes in the gastrointestinal tract of the Chinese mitten crab with PICRUST (a, individual level; b, average level)

that the dominant phyla were Tenericutes, Proteobacteria, Firmicutes, Chloroflexi and Actinobacteria by Illumina sequencing. However, Wang *et al.* (2018) reported that Proteobacteria, Firmicutes, Tenericutes and Bacteroidetes were “indigenous” flora of the crab, which is consistent with our results. These differences in the microbial communities in the GI tract of the Chinese mitten crab could reflect the different processing methods and the different environments from which the crabs were obtained. Cardona *et al.* (2016) indicated that the bacterial composition of the water influenced that of the intestine. Wang *et al.* (2018) indicated that the GI bacterial community was correlated to the bacterial populations of the source water, the environment bacteria in the pond.

The results of the present study demonstrated that the microbial community in the GI tract of the Chinese mitten crab was highly variable, which might be due to the differences in the environments, as discussed above, as well as the gender. Li *et al.* (2016) revealed that bacterial community in the GI tract of largemouth

bronze gudgeon showed significant differences between male and female fish. We also investigated the microbial communities of male and female Chinese mitten crabs, but we found no significant differences, which might due to the high variety of the GI microorganisms.

The microbial community varied among the samples in this study; however, the predicted functions demonstrated consensus and included Metabolism, Genetic Information Processing, Environmental Information Processing and Cellular Processes. This implied that the function of microorganisms in the GI tract was the same, although its community was affected by the environment. Cardiovascular diseases are reported as the leading cause of death in the world (Pagidipati and Gaziano, 2013). In the present study, the predicted function of cardiovascular diseases (Human Diseases) suggests that the crab should be well cooked before consumption as human food.

Conclusion

The present study provides an analysis of the gut microbial community and its predicted function in the GI tract of the Chinese mitten crab. These findings could aid in the control of the microbial community to improve the health and feed efficiency of this economically important crab.

Acknowledgements

The Research was supported by the China Agriculture Research System (No. CARS-46) and the Fundamental Research Funds from the Freshwater Fisheries Research Center (FFRC), Chinese Academy of Fishery Sciences, China (#2015JBFM03).

References

- Behrendt, L., A.W.D. Larkum, E. Trampe, A. Norman, S.J. Sorensen and M. Kuhl, 2012. Microbial diversity of biofilm communities in microniches associated with the didemnid ascidian *Lissoclinum patella*. *ISME J.*, 6: 1222–1237
- Caporaso, J.G., J. Kuczynski, J. Stombaugh, K. Bittinger, F.D. Bushman, E.K. Costello, N. Fierer, A.G. Pena, J.K. Goodrich, J.I. Gordon, G.A. Huttenhower, S.T. Kelley, D. Knights, J.E. Koenig, R.E. Ley, C.A. Lozupone, D. McDonald, B.D. Muegge, M. Pirrung, J. Reeder, J.R. Sevinsky, P.J. Turnbaugh, W.A. Walters, J. Widmann, T. Yatsunenko, J. Zaneveld and R. Knight, 2010. QIIME allows analysis of high-throughput community sequencing data. *Nat. Methods*, 7: 335–336
- Cardona, E., Y. Gueguen, K. Magre, B. Lorgeoux, D. Piquemal, F. Pierrat, F. Noguier and D. Saulnier, 2016. Bacterial community characterization of water and intestine of the shrimp *Litopenaeus stylirostris* in a biofloc system. *BMC Microbiol.*, 16: 157
- Chang, W., 2012. R graphics cookbook. O'Reilly Media, Inc., Sebastopol
- Di, P., X. Chen, G. Sun, J. Xiao and Y. Pan, 2014. Constitution analysis of intestinal dominant bacteria community in cultured Chinese mitten crab (*Eriocheir sinensis*). *J. Microbiol.*, 34: 58–61
- Ghanbari, M., H. Shahraki, W. Kneifel and K.J. Domig, 2016. A first insight into the intestinal microbiota of snow trout (*Schizothorax zarudnyi*). *Symbiosis*, 72: 183–193
- Hsieh, Y., C.M. Peterson, A. Raggio, M.J. Keenan, R.J. Martin, E. Ravussin and M.L. Marco, 2016. Impact of different fecal processing methods on assessments of bacterial diversity in the human intestine. *Front. Microbiol.*, 7: 1643
- Ji, Y., X. Kong, H. Li, Q. Zhu, Q. Guo and Y. Yin, 2017. Effects of dietary nutrient levels on microbial community composition and diversity in the ileal contents of pregnant Huanjiang mini-pigs. *PLoS One*, 12: e0172086
- van Kessel, M.A.H.J., B.E. Dutilh, K. Neveling, M.P. Kwint, J.A. Veltman, G. Flik and M.S.M. Jetten, 2011. Pyrosequencing of 16S rRNA gene amplicons to study the microbiota in the gastrointestinal tract of carp (*Cyprinus carpio* L.). *AMB Express*, 1: 41
- Langille, M.G., J. Zaneveld, J.G. Caporaso, D. McDonald, D. Knights, J.A. Reyes, J.C. Clemente, D.E. Burkepille, R.L. Thurber, R. Knight, R.G. Beiko and C. Huttenhower, 2013. Predictive functional profiling of microbial communities using 16S rRNA marker gene sequences. *Nat. Biotechnol.*, 31: 814–821
- Lewis, Z.T., K. Sidamonidze, V. Tsaturyan, D. Tsereteli, N. Khachidze, A. Pepoyan, E. Zhgenti, L. Tevzadze, A. Manvelyan, M. Balayan, P. Imnadze, T. Torok, D.G. Lemay and D.A. Mills, 2017. The fecal microbial community of breast-fed infants from Armenia and Georgia. *Sci. Rep.*, 7: 40932
- Li, K., W. Guan, G. Wei, B. Liu, J. Xu, L. Zhao and Y. Zhang, 2007. Phylogenetic analysis of intestinal bacteria in the Chinese mitten crab (*Eriocheir sinensis*). *J. Appl. Microbiol.*, 103: 675–682
- Li, X., Q. Yan, E. Ringo, X. Wu, Y. He and D. Yang, 2016. The influence of weight and gender on intestinal bacterial community of wild largemouth bronze gudgeon (*Coreius guichenoti*, 1874). *BMC Microbiol.*, 16: 191
- Liu, J., M. Zhang, C. Xue, W. Zhu and S. Mao, 2016. Characterization and comparison of the temporal dynamics of ruminal bacterial microbiota colonizing rice straw and alfalfa hay within ruminants. *J. Dairy Sci.*, 99: 1–14
- Meziti, A., E. Mente and K. Kormas, 2012. Gut bacteria associated with different diets in reared *Nephrops norvegicus*. *Syst. Appl. Microbiol.*, 35: 473–482
- Pagidipati, N.J. and T.A. Gaziano, 2013. Estimating deaths from cardiovascular disease: A review of global methodologies of mortality measurement. *Circulation*, 127: 749–756
- Rawls, J.F., B.S. Samuel and J.I. Gordon, 2004. Gnotobiotic zebrafish reveal evolutionarily conserved responses to the gut microbiota. *Proc. Natl. Acad. Sci. USA*, 101: 4596–4601
- Wang, C., Y. Zhou, D. Lv, Y. Ge, H. Li and Y. You, 2018. Change in the intestinal bacterial community structure associated with environmental microorganisms during the growth of *Eriocheir sinensis*. *Microbiologyopen*, 2018, Article No. e00727
- Yang, X., M. Xu, G. Huang, C. Zhang, Y. Pang and Y. Cheng, 2019. Effect of dietary L-tryptophan on the survival, immune response and gut microbiota of the Chinese mitten crab, *Eriocheir sinensis*. *Fish Shellfish Immunol.*, 84: 1007–1017

(Received 29 October 2018; Accepted 17 November 2018)