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Research Article

Carbohydrate Active Enzyme Profile of Bar-Headed Goose (*Anser indicus*) Gut Metagenome

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ABSTRACT

Carbohydrate Active enzymes (CAZymes), encoded by microbes that inhabit the gastrointestinal tract, play a crucial role in breakdown of complex dietary carbohydrates into components that can be absorbed by host intestinal epithelium. Bar-headed goose (*Anser indicus*), an herbivorous bird, has become one of the most popular wild birds for artificial rearing industries in several provinces of China. To understand how CAZymes in the gut of artificially reared Bar-headed geese are influenced by artificial diets, we describe here analysis of CAZymes from gut metagenomic data from both wild and artificially reared Bar-headed geese. A total of 172 and 215 CAZymes were found in wild and artificially reared Bar-headed geese, respectively. Glycoside hydrolases and glycosyl transferases were found to be the two most abundant categories of CAZymes in both groups. Comparative study showed a total of 22 significantly different CAZymes between wild and artificially reared group. Further, these significantly different CAZymes were observed to be specifically abundant in the *Firmicutes* phyla in wild group, whereas *Bacteroidetes* phyla in artificially reared group. These results provide a global view of CAZyme profiles of Bar-headed geese, and make an original contribution to the artificial diets management for rearing this bird.

Key words: Bar-headed goose, Gut metagenome, Carbohydrate active enzyme, High-throughput sequencing, Artificial rearing

INTRODUCTION

Advances in next-generation sequencing technologies coupled with new bioinformatic developments permit the study of the various microbiome (the complex collection of microorganisms, their genes, and their metabolites) of the human and animal bodies at an unprecedented scale (Human Microbiome Project, 2012, Qin *et al*., 2010, Weinstock, 2012). These microorganisms are no longer considered as disease-producing pathogens, rather they are now considered as a co-evolutionary partner contributing to both host health and disease (Backhed *et al*., 2005, Ley *et al*., 2008, Shapira, 2016). The gastro-intestinal tract contains the largest collection of micro-organisms, which are collectively named "gut microbiome". A growing number of studies have shown that gut microbiome play an important role in several fundamental and crucial processes such as host development (Malmuthuge *et al*., 2015), immune homeostasis (Ahern *et al*., 2014), nutrient assimilation (Kau *et al*., 2011), vitamins synthesis and sterols metabolism in the host (O'mahony *et al*., 2015), and diseases (e.g., obesity, diabetes, and cancer) in humans and other animals (Kinross *et al*., 2011, Lee and Hase, 2014).

The gut microbiota of herbivores is also the main driver of plant cell wall degradation as genomes of these animals do not encode most of the enzymes needed to degrade the structural polysaccharides present in plant material. In general and as expected, the gut microbiome of herbivorous animals encoded for high numbers of carbohydrate active enzymes (CAZymes). Thereby herbivores can gain 70% of their energy from microbial polysaccharide breakdown (Flint *et al*., 2012). CAZymes designate the ensemble of the enzymes that catalyze the assembly, breakdown or modification of oligosaccharides, polysaccharides and glycoconjugates. They are currently comprising 135 families of glycoside hydrolases (GHs), 24 polysaccharide lyases (PLs), 16 carbohydrate esterases (CEs), and 98 glycosyl transferases (GTs) (Lombard *et al*., 2014). In addition there are currently 64 families of

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carbohydrate binding modules (CBMs) that are indirectly associated with carbohydrate metabolism (Cockburn and Svensson, 2013), and in order to simplify our results and discussions, they will not be described in this manuscript.

The identification of these CAZymes is constrained due to the fact that the majority of gut microbes are uncultivable. Techniques for mining metagenomes have developed rapidly in recent years, and the huge output data are valuable resources for identifying putative CAZymes from both culturable and unculturable gut microbes (El Kaoutari *et al*., 2013, Vidal-Melgosa *et al*., 2015). Among avians, gut metagenome research mainly focused on commercially farmed species such as chicken (Sergeant *et al*., 2014), turkey (Lu and Domingo, 2008) and ostrich (Matsui *et al*., 2010). Only a very limited number of wild birds' metagenome have been reported in the literature (Roggenbuck *et al*., 2014, Waite and Taylor, 2015). However, very limited information is available for estimating gut microbes' functional activities because metagenome analyses tend to reflect bacterial composition, but not bacterial activity.

As one of the dominant waterfowl species in wetland areas in Qinghai-Tibetan Plateau, artificial rearing of Bar-headed geese (*Anser indicus*) is increasing in several provinces of China since year 2003 for the purpose of conservation and economic development. In our previous study (Wang *et al*., 2016b), we showed that the core gut microbiota of wild Bar-headed geese were dominated by *Firmicutes*, *Proteobacteria*, *Actinobacteria*, and *Bacteroidetes*. Furthermore, by comparative analysis of the gut microbiome of Bar-headed geese in different rearing conditions, we found that the artificially reared Bar-headed geese had significantly more *Bacteroidetes* compared to the wild ones (Wang *et al*., 2016a). As an herbivorous bird, the nourishment is composed of highly fibrous plant material, mainly grass, leaves, twigs and seeds (Middleton and van der Valk, 1987). However, the CAZymes profile of this species remains unknown.

Therefore, the first aim of the present study was to provide an overview of the CAZymes present in the gut microbiota of Bar-headed geese. To the best of the authors' knowledge, this is also the first data set to report CAZymes profile for the Bar-headed geese gut metagenome. The second aim was to evaluate the variation of CAZymes between wild Bar-headed geese group and artificially reared group.

MATERIALS AND METHODS

Ethics statement

This study was carried out in strict accordance with the Animal Management Rule of the National Health and Family Planning Commission, People's Republic of China (Documentation 55, 2001). The research protocol was reviewed and approved by the Animal Care and Use Committee of the Chinese Academy of Sciences. The manuscript does not contain experiments using animals and human studies.

Faecal samples collection

Two groups of faecal samples were obtained in Qinghai province, China, on 2nd July, 2014. The wild Bar-headed geese faecal samples (n = 2) were collected from Ha Datan wetland (37°07'41.3"N, 99°43'39.9"E, elevation 3,100 m). The artificially reared (abbreviation: AR) Bar-headed geese faecal samples (n = 2) were derived from Bu Ha River Estuary (36°58'25.5"N, 99°50'19.2"E, elevation 3,197 m) in Qinghai Lake. The AR populations lived freely in both wild and captivity environments, fed on both natural and artificial diets (commercial blended feed for chicken). These populations were not treated with antibiotics. About 1 g of faecal samples were collected from faecal balls, avoiding collection of faecal material that was touching the ground. All samples were placed in sterile containers and transported to the laboratory in car-carried refrigerator. In laboratory, faecal samples were kept frozen at -80°C until processing.

DNA extraction and shotgun metagenomic sequencing

Genomic DNA was isolated from approximately 1 g of faecal sample using the E.Z.N.A. ® stool DNA Kit (Omega Bio-tek, Norcross, GA, USA) following the manufacturer's instruction. DNA concentration and quality were determined using QuantiFluor™ - ST (Promega, Madison City, WI, USA) and gel electrophoresis respectively. With the extracted DNA, library construction was performed on an Illumina Hiseq2500 platform according to the standard protocols. Metagenome sequences data are now available at NCBI under the Sequence Read Archive (SRA) database with accession No.SRP072790 and No.SRP072793.

Bioinformatic analysis of sequencing data

Raw sequences obtained from 4 metagenomic samples were subjected to a quality check using the FastQC software (version v0.11.3) (Andrews, 2012). All samples showed satisfactory values for each parameter tested. Next, the sequences were run through Trimmomatic (version 0.33) (Bolger *et al*., 2014) to remove low quality base pairs using these parameters (SLIDINGWINDOW: 4:15 MINLEN: 36). Further, the host specific and other eukaryotic sequences were removed by parsing the NCBI non-redundant protein database (NCBI-nr) taxonomy assignment using the lowest common ancestor (LCA) algorithm in MEGAN (Huson *et al*., 2007). The protein-encoding open reading frame (ORF) from these resulting cleaned sequences were predicted using Prodigal (version 2.6.2) (Hyatt *et al*., 2012). CAZymes were identified from these protein coding sequences using dbCAN (Yin *et al*., 2012), a web resource that implements hidden Markov models (HMMs) for automated signature domain-based annotations representative of each individual category and family. For the dbCAN assignments, a minimum e-value cut-off of 1×10^{-3} was used. Two-sided Welch's t-test (Parks and Beiko, 2010) in STAMP software package was applied to test the CAZymes categories differences between the AR and Wild group. Categories difference with a p value of <0.05 were considered to be significant. All figures were generated with customized R scripts.

RESULTS

Diversity profile of CAZymes found in Bar-headed geese gut metagenome

In the wild group, a total of 172 CAZymes were found (Table S1). They were 14 CEs (4,188 reads), 93

GHs (30,896 reads), 50 GTs (11,356 reads) and 15 PLs (246 reads). A total of 215 CAZymes were found in the AR group (Table S2), including 16 CEs (30,950 reads), 116 GHs (331,970 reads), 64 GTs (77,767 reads) and 19 PLs (9,687 reads). The average proportion of each class of the CAZymes revealed higher proportion of GHs and GTs in both groups. The average detection frequency of these totally five CAZymes among the total clean reads generated from gut metagenome was 0.94% and 1.91% in the wild and AR group, respectively (Table S3).

Comparison of CAZymes between wild and artificially reared Bar-headed geese

The proportion of each CAZymes was tested statistically with STAMP using Welch's t-test. As shown in Figure 1, GH73, 90, 119, 126 and GT45, 96 were found to be significantly higher in wild group ($P<0.05$) compared to AR group. Another 16 CAZymes had higher proportions in AR group ($P<0.05$), including GH10, 30, 35, 51, 53, 55, 77, 82, 84, 98, 121, 127, 130, PL11, 12 and CE12.

Phylogenetic classification of significantly different CAZymes

These 22 significantly different CAZymes were further analyzed for their microbial origin. In the wild group, all the 6 CAZymes were highly enriched in phylum *Firmicutes* (Table 1). In the AR group, 15 CAZymes were found to be enriched in phylum *Bacteroidetes*, while GH77 were found to be only highly enriched in phylum *Firmicutes* (Table 2). These results indicate that different types of gut microbes contribute differently to the occurrence of CAZymes.

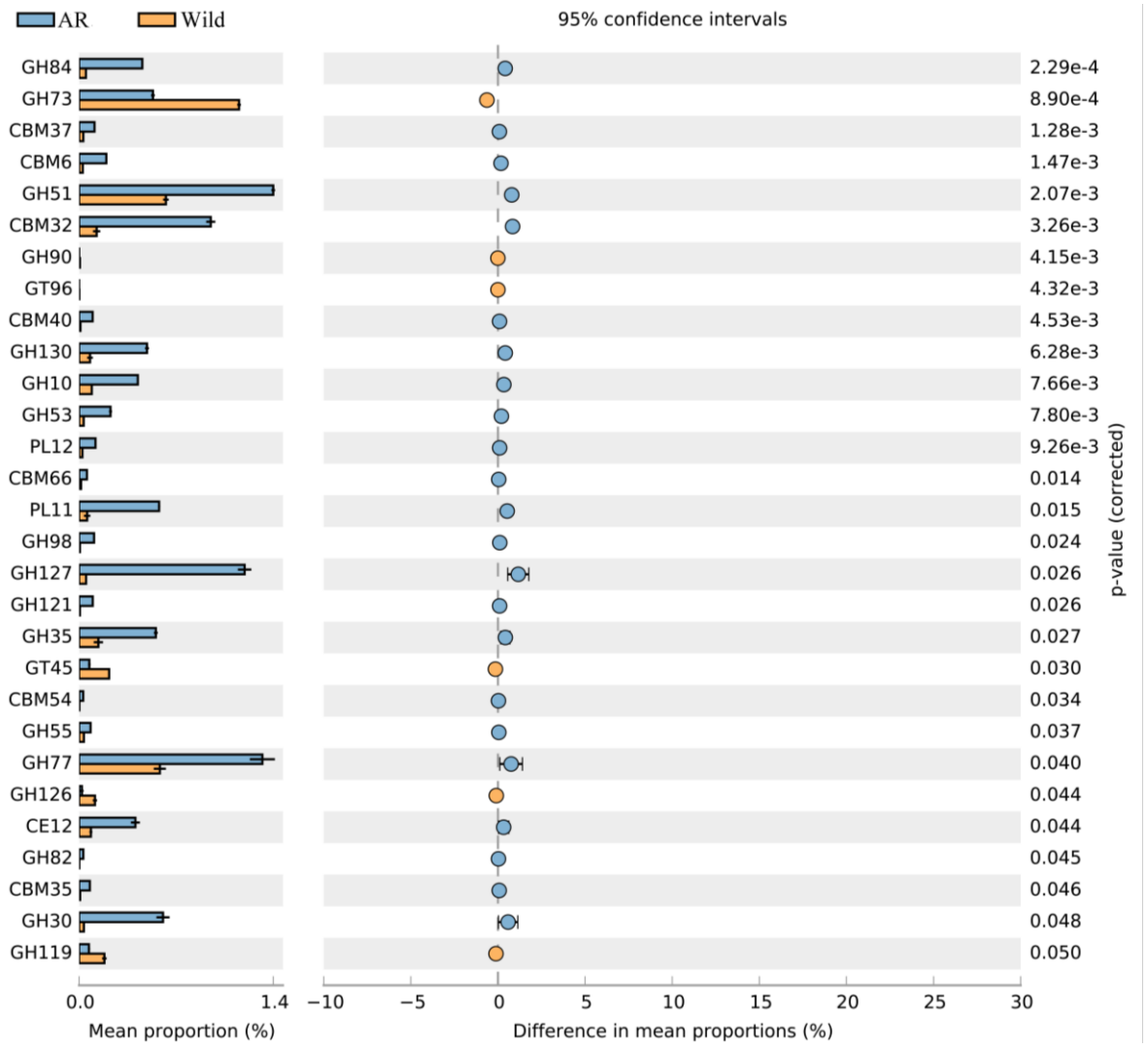

Fig. 1: Significant CAZymes differences as a result of Welch's t-test between the AR and Wild group conducted with the STAMP program. Difference with a *p* value of <0.05 were considered to be significant.

Table 1: The taxonomic assignment at the phylum level of each significantly higher CAZymes in wild group

CAZymes	Known activities	Corresponding phylum (average propotion)
GH73	mannosyl-glycoprotein endo-β-N-acetylglucosaminidase	Firmicutes (80.45%)
GH90	endorhamnosidase	Firmicutes (83.335%)
GH119	α-amylase	Firmicutes (63.72%)
GH126	α-amylase	Firmicutes (99.00%)
GT45	α-N-acteylglucosaminyltransferase	Firmicutes (92.96%)
GT96	peptidyl serine α-galactosyltransferase	Firmicutes (100.00%)

Table 2: The taxonomic assignment at the phylum level of each significantly higher CAZymes in AR group

CAZymes	Known activities	Corresponding phylum (average propotion)
GH10	endo-1,4-β-xylanase	Bacteroidetes (77.84%)
GH30	endo-β-1,4-xylanase	Bacteroidetes (76.81%)
GH35	β-galactosidase	Bacteroidetes (83.99%)
GH51	endo-β-1,4-xylanase	Bacteroidetes (71.14%)
GH53	endo-β-1,4-galactanase	Bacteroidetes (85.14%)
GH55	endo-β-1,3-glucanase	Bacteroidetes (62.47%)
GH77	amylomaltase or 4-α-glucanotransferase	Firmicutes (48.47%)
GH82	I-carrageenase	Bacteroidetes (91.56%)
GH84	N-acetyl β-glucosaminidase	Bacteroidetes (67.04%)
GH98	endo-β-1,4-xylanase	Bacteroidetes (76.71%)
GH121	β-L-arabinobiosidase	Bacteroidetes (66.25%)
GH127	β-L-arabinofuranosidase	Bacteroidetes (72.74%)
GH130	β-1,4-mannosylglucose phosphorylase	Bacteroidetes (78.13%)
PL11	rhamnogalacturonan lyase	Bacteroidetes (79.18%)
PL12	heparin-sulfate lyase	Bacteroidetes (68.98%)
CE12	pectin acetylesterase	Bacteroidetes (87.96%)

Table S1: List of all CAZymes that were found in wild Bar-headed geese gut metagenomic data sets

CAZymes	Wild_1	Wild_2	CAZymes	Wild_1	Wild_2	CAZymes	Wild_1	Wild_2	CAZymes	Wild_1	Wild_2
CE1	1153	1060	GH133	2	1	GH70	127	112	GT41	198	170
CE10	360	343	GH14	70	64	GH71	10	5	GT43	1	1
CE11	118	149	GH15	36	73	GH73	625	587	GT44	1	1
CE12	41	48	GH16	30	32	GH74	75	107	GT45	120	108
CE13	2	4	GH17	36	46	GH76	17	12	GT46	7	3
CE14	86	104	GH18	165	135	GH77	295	315	GT48	1	4
CE2	14	9	GH19	53	62	GH78	24	29	GT5	1336	1195
CE3	26	33	GH2	1520	1443	GH79	6	2	GT51	1932	1852
CE4	760	692	GH20	106	138	GH8	309	282	GT52	2	9
CE5	9	9	GH22	1	1	GH82	1	1	GT53	4	5
CE6	20	29	GH23	339	320	GH84	29	23	GT55	5	4
CE7	159	163	GH24	67	59	GH85	37	30	GT56	3	5
CE8	31	35	GH25	573	500	GH87	2	9	GT62	1	10
CE9	1523	1397	GH26	2	2	GH88	10	28	GT66	3	1
GH1	12924	11142	GH27	10	5	GH9	9	19	GT7	4	4
GH10	50	46	GH28	16	39	GH90	3	3	GT70	3	1
GH100	2	1	GH29	129	175	GH92	161	176	GT73	5	11
GH101	20	33	GH3	992	813	GH94	816	688	GT76	4	4
GH102	36	40	GH30	15	21	GH95	63	88	GT8	80	70
GH103	183	198	GH31	224	241	GH97	26	83	GT81	132	114
GH104	4	9	GH32	1394	1311	GH99	2	2	GT83	143	173
GH105	38	58	GH33	49	65	GT1	39	29	GT84	2	8
GH106	14	21	GH35	58	87	GT10	14	20	GT87	1	4
GH108	5	5	GH36	1518	1357	GT11	103	103	GT9	57	78
GH109	604	598	GH37	6	10	GT12	8	10	GT90	2	1
GH11	5	7	GH38	400	350	GT14	13	13	GT92	41	37
GH110	1	4	GH39	19	24	GT17	12	5	GT94	15	23
GH111	1	1	GH4	411	332	GT19	130	159	GT96	1	1
GH112	7	3	GH42	882	764	GT2	2860	2671	PL1	14	12
GH113	35	44	GH43	362	343	GT20	46	58	PL10	3	6
GH114	12	12	GH46	1	1	GT21	4	9	PL11	19	41
GH115	105	95	GH48	13	20	GT25	10	8	PL12	11	14
GH116	2	2	GH5	32	28	GT26	169	154	PL14	2	2
GH117	20	18	GH50	108	133	GT27	35	47	PL16	1	2
GH119	91	100	GH51	354	305	GT28	493	494	PL17	1	1
GH121	2	4	GH53	22	13	GT3	1	7	PL2	1	1
GH123	3	4	GH55	17	19	GT30	62	66	PL22	21	23
GH125	141	140	GH57	6	10	GT31	4	8	PL3	1	1
GH126	71	50	GH63	8	13	GT32	86	89	PL5	63	64
GH127	30	23	GH65	1338	1168	GT35	2021	1732	PL6	6	5
GH129	23	32	GH66	7	8	GT39	21	28	PL7	29	38
GH13	3575	3188	GH67	319	236	GT4	1470	1372	PL8	14	21
GH130	32	50	GH68	59	44	GT40	16	12	PL9	48	27

Table S2: List of all CAZymes that were found in artificially reared Bar-headed geese gut metagenomic data sets

CAZymes	AR_1	AR_2	CAZymes	AR_1	AR_2	CAZymes	AR_1	AR_2	CAZymes	AR_1	AR_2
CE1	1685	8766	GH16	362	3854	GH76	104	953	GT41	726	6773
CE10	736	3109	GH17	33	16	GH77	1739	11936	GT42	2	15
CE11	451	4417	GH18	331	2341	GH78	1154	9814	GT44	5	16
CE12	528	3704	GH19	135	150	GH79	50	222	GT45	117	550
CE13	4	3	GH2	9380	66679	GH8	305	706	GT46	2	34
CE14	236	615	GH20	3096	26503	GH80	2	16	GT5	2303	9889
CE15	11	182	GH22	4	1	GH81	9	293	GT50	3	1
CE16	1	1	GH23	698	4430	GH82	46	241	GT51	2387	8829
CE2	105	1202	GH24	186	1012	GH84	650	3819	GT52	7	1
CE3	107	708	GH25	1068	6087	GH85	50	512	GT53	71	35
CE4	1261	5931	GH26	286	2177	GH86	5	20	GT55	6	3
CE5	29	31	GH27	216	3358	GH87	37	349	GT56	9	61
CE6	244	2135	GH28	778	5841	GH88	1028	7920	GT58	1	11
CE7	566	3291	GH29	2247	22188	GH89	1000	15336	GT59	2	3
CE8	340	2442	GH3	4081	26513	GH9	114	1010	GT6	24	91
CE9	3294	15765	GH30	788	5513	GH90	1	2	GT62	4	10
GH1	11504	13896	GH31	2324	17802	GH91	16	128	GT64	1	12
GH10	604	3548	GH32	3074	11953	GH92	4355	42105	GT66	8	52
GH100	3	25	GH33	614	6692	GH93	17	247	GT7	20	412
GH101	125	710	GH34	1	16	GH94	930	2954	GT70	2	30
GH102	48	96	GH35	762	4795	GH95	2576	21042	GT73	8	60
GH103	231	298	GH36	4419	20303	GH97	4051	36640	GT76	14	44
GH104	4	7	GH37	202	735	GH98	155	896	GT8	327	2925
GH105	924	8331	GH38	519	1220	GH99	16	284	GT81	188	979
GH106	296	4800	GH39	107	733	GT1	86	531	GT82	1	39
GH107	1	15	GH4	617	1659	GT10	29	1282	GT83	294	1872
GH108	71	288	GH42	1386	4602	GT11	125	1063	GT84	11	432
GH109	1142	5015	GH43	3437	26878	GT12	27	188	GT85	26	10
GH11	9	22	GH44	13	478	GT13	14	357	GT87	37	31
GH110	543	3833	GH46	3	6	GT14	77	1002	GT89	25	6
GH111	16	50	GH47	7	144	GT15	2	22	GT9	168	1070
GH112	866	2897	GH48	18	9	GT17	6	21	GT90	13	128
GH113	66	181	GH49	75	394	GT18	1	2	GT92	83	867
GH114	24	21	GH5	233	2853	GT19	554	4879	GT94	42	265
GH115	1374	10313	GH50	172	284	GT2	4995	35222	PL1	245	1733
GH116	61	1274	GH51	1958	11945	GT20	181	320	PL10	112	1458
GH117	340	3134	GH52	1	5	GT21	21	127	PL11	803	4946
GH118	1	4	GH53	305	2009	GT22	4	24	PL12	175	949
GH119	112	529	GH55	118	695	GT23	35	266	PL13	3	69
GH120	79	319	GH57	130	2548	GT25	40	63	PL14	4	12
GH121	130	884	GH59	26	116	GT26	311	2205	PL15	27	268
GH123	280	1297	GH6	19	17	GT27	96	820	PL16	5	6
GH124	1	23	GH62	4	25	GT28	956	4979	PL17	14	178
GH125	378	1703	GH63	134	1173	GT29	1	7	PL21	24	205
GH126	46	66	GH64	3	8	GT3	443	6157	PL22	181	1492
GH127	1754	9680	GH65	1238	2037	GT30	196	1289	PL23	1	8
GH128	45	485	GH66	32	1149	GT31	6	46	PL3	2	10
GH129	72	270	GH67	679	3594	GT32	178	1309	PL4	86	885
GH13	5435	19807	GH68	43	11	GT33	5	33	PL5	58	13
GH130	711	4025	GH70	114	143	GT35	3513	15302	PL6	75	629
GH132	1	2	GH71	9	6	GT37	5	33	PL7	36	7
GH133	431	5245	GH72	3	68	GT39	137	613	PL8	342	3189
GH14	46	12	GH73	733	4613	GT4	2877	19570	PL9	78	1046
GH15	118	340	GH74	214	931	GT40	41	348			

Table S3: Summary of clean reads and CAZymes reads obtained from gut metagenomic data sets of each sample

Samples	Total clean reads	CAZymes reads
Wild_1	5,019,282	51,106
Wild_2	5,431,620	46,815
AR_1	7,582,586	134,003
AR_2	39,269,524	809,155

DISCUSSION

It is widely acknowledged that the vertebrate gut microbiome play critical roles in host health and disease (Zhang *et al*., 2015), which is now attracting increasing attention in the wild birds research (Waite and Taylor, 2014). Metagenome sequencing approaches, which rapidly produce millions of whole genome shotgun sequencing reads that enable the investigation on a culture independent basis, are now popular for exploring microbial community (Jovel *et al*., 2016). In the present study, for the first time, we outline CAZymes profile of Bar-headed goose metage-nome and we find variations of these CAZymes between wild Bar-headed geese group and artificially reared group.

Our previous studies have demonstrated that the variations in Bar-headed geese gut microbiota diversity

and structure is mostly due to the rearing conditions (Wang *et al*., 2016a). Furthermore, in the present study, the different gut microbes contribute to the generation of different CAZymes. In the wild group, the significantly increased CAZymes totally belonged to *Firmicutes* phyla, while the chief contributors of the significantly increased CAZymes in the AR group are *Bacteroidetes*. In *Firmicutes*, degradative capacity is largely restricted to the cell surface and involves elaborate cellulosome complexes in specialized cellulolytic species. By contrast, in the *Bacteroidetes*, utilization of soluble polysaccharides entails outer membrane binding proteins, and degradation is largely periplasmic or intracellular. In general, *Bacteroidetes* are well reported for their starch, pectin and xylan digestion (Thomas *et al*., 2011), while *Firmicutes* for their cellulose and hemicellulose digestion (Flint *et al*., 2012). Further, previous study has shown presence of higher saccharolytic potential in *Bacteroidetes* as compared to *Firmicutes* (El Kaoutari *et al*., 2013). The AR populations had unrestricted access to fly away to seek natural food and were also fed on artificial diets (blends of 60% corn flour, 20% soybean flour and some vegetables). The members of phylum *Bacteroidetes* may therefore confer more efficient extraction of energy from both natural and artificial food resources for the artificially reared Bar-headed geese. The microbial communities of the wild Bar-headed geese (Wang *et al*., 2016a) were mainly dominated by *Firmicutes* (60.67%), with very low relative abundances of *Bacteroidetes* (3.33%). Therefore, the digestion of dietary polysaccharides contained in natural diets was mainly conducted by the CAZymes produced by the *Firmicutes* phyla.

GHs are modular enzymes consisting of different combinations of catalytic modules and helper modules. About 1% of the genome of any organism encodes for GHs (Vocadlo and Davies, 2008). Of the total detected GHs, the proportions of 4 GHs were significantly increased in wild group, with the capacity of digestion cellulose (Flint *et al*., 2008). 13 GHs were found to be significantly increased in AR group. 4 of them (GH10, 30, 51, 98) are xylanases involved in xylan breakdown. GH35, 53, 55, 77, 84 and PL11 enzymes encompass beta-galactosidases, beta-glucosidases. These enzymes are involved in the breakdown of a large variety of oligosaccharides. Taken together, these increased CAZymes in each group indicate enrichment of gut microbes harboring CAZymes and thereby playing beneficial role in utilization of complex plant polysaccharides.

This data set is not without limitations. First, the number of samples used for sequencing was low. Second, the content of artificial diets as compared to natural diets was not precisely defined. The relationship among different dietary elements, gut microbes and CAZymes should be documented in the future. At this time, however, this is the most comprehensive data set of its type, and provides more knowledge of the Bar-headed geese microbiome and how it may be impacted by dietary intervention.

Conclusions

In conclusion, the present study reveals the global picture of CAZyme profiles of both wild and artificially reared Bar-headed geese. This study can form a basis for further investigations into CA Zyme profiles and the gut microbes harboring them, on much larger flocks of Bar-headed geese.

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