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Comparative Analysis of Intestinal Microbiota Diversities in Four Chinese Local Pig Breeds and Landrace Pig

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Abstract: Iatric obesity is a growing problem around the world with an increasing prevalence among even infants and young children. The intestinal microbiota have recently been shown to affect the energy balance by influencing both the efficiency of calorie harvest from the diet and how harvested energy is used and stored and is a non-negligible contributor of the formation of obesity. Pig is a good model for use in human nutrition research but few studies have examined the differences among different pig breeds. This study was conducted to compare the diversities of intestinal microbiota among lean Landrace pig and four local Chinese pig breeds with obese phenotype including Bama mini-pig, Huanjiang mini-pig, Ningxiang pig and Lantang pig. These local Chinese pig breeds have obvious differences in genotype and phenotype compared to Landrace pig. The aim of this study was to examine the association between the intestinal microbiota composition in infancy and future obesity using pigs as a model. The results indicated that the local Chinese pig breeds had significantly greater microbiota diversities in the distal intestine than Landrace pig. There are also clear differences in genus that can influence the energy balance and contribute to obesity. The results indicated that the microbiota diversity in the distal intestine in LCBs was significant higher than in LD which challenge the result that the microbiota diversity decrease in obese individuals. Intestinal microbiota do contribute for the obese phenotype but genotype is the main contributor. The higher contents of intestinal microbial populations that show strong energy-harvesting ability may partly contribute to the obese phenotype in local Chinese pigs. The research may help to clarify the mechanism of phenotype diversity in pig breeds and contribute to studies on infant nutrition and obesity.

Key words: Energy metabolism, intestinal microbiota, local Chinese pig breed, obesity, population

INTRODUCTION

Nowadays, obesity is more prevalent around the world and is becoming one of the important public health problems in the times. The highest morbidity is in children (Owen *et al*., 2005; He *et al*., 2012). Childhood obesity is dangerous and will bring increased risks for other diseases including diabetes, arterial hypertension, coronary artery disease, fatty liver and other metabolic diseases (Barton, 2012). As early as 150 years ago, childhood obesity had been one of medical interests (Don, 1859; Ellis and Tallermann, 1934) and now it is still be focused in obesity researches (Mazur *et al*., 2013). The formation mechanism of obesity is very complex there are various factors that identified be associated with childhood obesity (Putre, 2013; Sun *et al*., 2013). Among these factors, intestinal microbiota is a non-negligible contributor (Vael and Desager, 2009). It was well known that the mammalian distal intestinal harbors a vast ensemble of intestinal microbiota and which have a profound influence on physiology and nutrition and which are crucial for life (Backhed *et al*., 2005; Hooper *et al*., 2002). They affect the host metabolism and improve the ability to extract energy from indigestible dietary polysaccharides and nutrient harvest (Ley *et al*., 2005, 2006; Jumpertz *et al*., 2011). Recent studies noted a correlation between intestinal microbiota and obesity (Eckburg *et al*., 2005; Turnbaugh *et al*., 2006). The

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metabolic activity of intestinal microbiota in obese children is increased compared with normal-weight children and exhibits more exhaustive substrate utilization (Payne *et al*., 2011). But in most of these studies, results were summarized based on analysis of feces samples which could not reflect the real station in the intestine. Certainly, it was hard to get the human intestinal contents and most of studies choose mice or rat as the animal models. However, mice or rat has distant phylogenetic relationships and have large difference in characteristics of digestion and absorption. Pig is a good model for use in human nutrition research (Miller and Ullrey, 1987) which is quite similar to humans with respect to the anatomy, physiology and metabolism of the digestive system (Pang *et al*., 2007) and has strong capacity for fat storage, especially in native Chinese species. Local Chinese pig Breeds (LCBs) mainly comprised by obese strains and their muscle and fat developments is different from that of Landrace pig (LD) (Zhao *et al*., 2011; He *et al*., 2012). Even among LCBs there are obvious differences in genotype and phenotype. In contrast to commercial pig breeds (e.g., LD) these LCBs are mainly fed in the traditional Chinese way with most of their food with green roughage. Over thousands of years they have adapted to the feed with low protein and high fiber contents. Obese and lean pigs of the Banna mini-pig inbred line have been used in a comparative study of microbiota (Guo *et al*., 2008a, b). Researchers surmised that the composition of intestinal microbiota should have different composition among LCBs and LD based on their ability to thrive on a low protein and high fiber diet and this difference may contribute to the obese phenotype of LCBs. The aim of this study was to compare the intestinal microbiota composition in Landrace pig (LD) and four LCBs including Bama Mini-pig (BM), Huanjiang mini-pig (HJ), Lantang pig (LT) and Ningxiang pig (NX) and the result may help to clarify mechanism of phenotype diversity in pig breeds and contribute to studies on childhood obesity.

MATERIALS AND METHODS

Intestinal contents sampling: Four LCBs (BM, HJ, LT and NX) and LD were used in the present study. Detailed information on sampling location is shown in Table 1. For each pig breed, 6 males and 6 females weanling piglets were selected from two litters. All of the sampled piglets were nursed by sows in their provenances before they were slaughtered (Liu *et al*., 2012a). At age of 14 days, the sampled piglets were sacrificed by jugular puncture under general anesthesia via the intravenous injection of 4% sodium pentobarbital solution (40 mg kg^{-1} B.W.) and then immediately eviscerated (Liu *et al*., 2012b). The intestinal contents were sampled from the ileum, cecum and colon using sterile plastic tubes and stored at -80°C until analysis (Deng *et al*., 2009). The entire process from defecation to storage did not exceed 1 h (Yin *et al*., 2010). All experimental procedures used in this study were approved by the Animal Care and Use Committee of the Chinese Academy of Sciences (Kong *et al*., 2007; Yao *et al*., 2012).

Table 1: Detailed information on sampling places of Chinese local pig breeds used in this study

Local pig breeds	Longitude	Latitude	Elevation (M)
Huangjiang mini-pig	108.27'40.8"	25.9'50"	578
Bama mini-pig	107.13'5.4"	24.9'58.9"	242
Taoyuan black pig	111.27'35.4"	28.56'52.4"	65
Ningxiang pig	112.4'58.9"	28.1'47.7"	174
Lantang pig	114.11'30.3"	23.57'30.2"	482

DNA isolation from intestinal content samples: Metagenomic DNA was extracted from 250 mg intestinal contents sampled from ileum, cecum and colon (Tan *et al*., 2011) using the QIAamp DNA Stool Minikit (QiagenGmbH, Hilden, Germany) according to the manufacturer's instructions. Purified DNA was quantified by spectrophotometry using NanoDrop® ND2000 (NanoDrop Technologies Inc., DE, USA).

Community fingerprinting of intestinal microbiota by PCR-DGGE: The purified DNA (25 ng μL^{-1}) was used as template to amplify the variable V3 *16S rRNA* gene sequence by PCR reaction using 20 nM universal primers HAD-1-GC-f(CGCCCGGGGCGCGCCCCGGGCG GGGCGG GGGCACGGGGGGACTCCTACGGGAGGCAGC AG) and HAD-2-r (GTATTACCGCGGCTGCTGGCA) (Walter *et al*., 2000). PCR reactions consisted of 2x Fermentas PCR Mastermix diluted 1:1 with sterile ultra-pure water. Samples were amplified on an Eppendorf mastercycler (Gottingen, Germany) with the following parameters: 94°C for 4 min, 30 cycles of 94°C for 30 sec, 56°C for 30 sec, 72°C for 2 min and finally 72°C for 10 min and then held at 4°C. The PCR products were checked by electrophoresis on 2% agarose and then stored at -20°C until for Denaturing Gradient Gel Electrophoresis (DGGE) analysis. DGGE was carried out using the D-Code Universal Mutation Detection System (Bio-Rad, Hercules, CA). The PCR products were loaded at 20 μL per lane on 8% (wt/vol) polyacrylamide gels (37.5:1, acrylamide: bisacrylamide) in 1×Tris acetate EDTA buffer (40 mM Tris, 20 mM acetic acid, 1 mM disodium EDTA). Gels had a denaturing gradient ranging from 35-65% where 100% denaturant contained 7 mol L^{-1} urea and 40% deionized formamide. Electrophoresis was performed at 60°C, 200 V for 10 min and then 120 V for 16 h. Gels were stained according to the rapid silver-staining procedure (Sanguinetti *et al*., 1994) and photographed with a molecular imager®ChemiDoc™XRS+instrument (Bio-Rad). Electrophoretic profiles were analyzed using QuantityOne 4.62 Software (Bio-Rad) and similarity dendrograms were

generated via an Unweighted Pair Group Method using Arithmetic averages (UPGMA) clustering (Wang *et al*., 2011).

Random cloning and sequencing of *16S rDNA* genes: Full-length regions of the bacterial *16S rDNA* gene pools were amplified by PCR using the universal primers F8 (AGAGTTTGATCCTGGCTCAG) and R1492 (GGTTACCTTGTTACGACTT) (Eden *et al*., 1991). The PCR mixture (50 μL in total) contained 5 μL 10×buffer, 10 ng metagenomic DNA, 1 μL (10 nM) of each primer and 2.5U pfu DNA polymerase (TaKaRa, Dalian, China). The PCR reaction was performed by using an Eppendorf mastercycler (Gottingen, Germany) with the following parameters: initial denaturation at 95°C for 5 min, 20 cycles of denaturation at 95°C for 30 sec, annealing at 56°C for 30 sec and elongation at 72°C for 1.5 min and final elongation at 72°C for 10 min. Four 5 μL of the PCR products were identified by electrophoresis at 120 V for 50 min on a 1% agarose gel. The amplification products were purified using a Promega Wizard SV Gel and PCR Clean-up System (Promega Corporation, WI, USA) and then be linked to pGEM-T Easy (Promega Corporation, WI, USA). The plasmids were introduced into competent DH5α cell and incubated overnight at 37°C. Cells that contained successfully constructed plasmids were obtained using an X-Gal screening test.

The plasmids were got using EZ-10 Spin Column Plasmid DNA Minipreps kit (BBI, Shanghai, China) according to the recommend protocol and then be sequenced at BGI (Beijing, China). The nearly full-length *16S rDNA* gene sequences were directly compared with corresponding sequences in GenBank using BLASTN. The cloned sequences were aligned by Clustal X. The aligned sequences were exported to Phylip packages where the phylogenetic trees of all of the sequences were reconstructed by a neighbor-joining algorithm (Galtier *et al*., 1996). The reliability of internal branches was assessed using 1000 bootstraps. The aligned sequences were grouped into Operational Taxonomic Units (OTUs). An OTU was defined to contain sequences with less than a 3% difference as detected by the furthest-neighbor method.

Real-time PCR analysis: Real-time PCR was performed to further quantitatively analyze the subpopulation of intestinal microbiota using group-specific primers that targeted a total of 16 genera which were previously reported to be associated with energy harvesting, including Bacteroidetes, Firmicutes, Bifidobacterium, *Bacteroides thetaiotaomicron*, *Clostridium coccoides* group, *Clostridium coccoides eubacteria*, *Clostridium leptum* subgroup, *Clostridium perfringens* group, Escherichia, *Faecalibacterium prausnitzii*, *Fusobacterium prausnitzii*, *Methanobrevibacter smithii*,

Table 2: Group and species-specific *16S rRNA* gene-targeted primers used in this study

Genus	Primers sequence	Length
All bacteria	F: TCCTACGGGAGGCAGCAGT	466
	R: GACTACCAGGGTATCTAATCCTGTT	
Bacteroidetes	F: AGCAGCCGCGGTAAT	184
	R: CTAHGCATTTCACCGCTA	
Bacteroides thetaiotaomicron	F: GGCAGCATTTCAGTTTGCTTG	423
	R: GGTACATACAAAATTCCACACGT	
Firmicutes	F: GTCAGCTCGTGTCGTGA	179
	R: CCATTGTAKYACGTGTGT	
Staphylococcus aureus	F: GCGATTGATGGTGATACGGTT	-
	R: AGCCAAGCCTTGACGAACTAAAGC	
Clostridium coccoides group	F: AAATGACGGTACCTGACTAA	440
	R: CTTTGAGTTTCATTCTTGCGAA	
Clostridium leptum subgroup	F: GCACAAGCAGTGGAGT	239
	R: CTTCCTCCGTTTTGTCAA	
Clostridium perfringens group	F: ATGCAAGTCGAGCGA(G/T)G	120
	R: TATGCGGTATTAATCT(C/T)CCTTT	
Clostridium coccoides	F: CGGTACCTGACTAAGAAGC	429
Eubacteria rectale group	R: AGTTTYATTCTTGCGAACG	
Prevotella	F: CACRGTAAACGATGGATGCC	513
	R: GGTCGGGTTGCAGACC	
Escherichia coli	F: GACCTCGGTTTAGTTCACAGA	585
	R: CACACGCTGACGCTGACCA	
Faecalibacterium prausnitzii	F: GGAGGAAGAAGGTCTTCGG	248
	R: AATTCCGCCTACCTCTGCACT	
Fusobacterium prausnitzii	F: CCCTTCAGTGCCGCAGT	158
	R: GTCGCAGGATGTCAAGAC	
Peptostreptococcus productus	F: AACTCCGGTGGTATCAGATG	268
	R: GGGGCTTCTGAGTCAGGTA	
Methanobrevibacter smithii	F: CCGGGTATCTAATCCGGTTC	123
	R: CTCCCAGGGTAGAGGTGAAA	
Roseburia	F: TACTGCATTGGAAACTGTCG	230
	R: CGGCACCGAAGAGCAAT	

Peptostreptococcus, Prevotella, Roseburia and *Staphylococcus aureus*. Quantitative PCR was carried out using genus and group-specific primers that targeted *16S rDNA* genes (Table 2). Amplification and detection of DNA by qPCR was performed with ABI PRISM 7900 HT (Applied Biosystems) using 384 well plates. Duplicate sample analysis was routinely performed in a total volume of 25 μL using SYBR® Premix Ex Taq™ II (TAKARA, Dalian, China) following the suggested protocol. Data from duplicate samples were analyzed using the ABI 7900 SDS Software (Version 2.3, Applied Biosystems).

Statistical analysis: For each sample, the results were normalized by referring to the average bacteria amplified CT of all extracted DNA. The results are expressed as the mean±SEM. Differences between more than two independent groups were analyzed by Fisher's test after ANOVA using SAS9.2 (SAS Institute Inc., Cary, NC). The level of significance was set at 0.05. Figures were made using Origin 8.0.

RESULTS

Clear difference exist among intestinal microbial community profiles of LCBs and LD: The diversity of intestinal microbiota in the samples were analyzed using DGGE and evaluated with band number. The results are shown in Fig. 1 and 2. Overall, the LCBs have obviously higher intestinal microbiota diversity than LD. Further, mini pigs (BM and HJ) have higher intestinal microbiota diversity than the other tested LCBs. There is also an interesting result that LT have a lower intestinal microbiota diversity in ileum and colon than LD. From the dendrogram of DGGE profils researchers will find the caecal microbiota composition of LD is most similar to the microbiota composition in colon of LCBs, the colonic microbiota composition of NX is most similar to the microbiota composition in cecum of other LCBs. To further clarify the intestinal microbiota composition, random cloning and *16S rDNA* genes sequencing were carried out. According with the results of DGGE, the results show that LCBs have a more complex microbiota composition. Mini pigs (BM and HJ) have higher intestinal microbiota diversity than the other tested LCBs. The details intestinal microbiota were shown in Fig. 3-5.

Shifts in the intestinal microbiota community with energy exaction from chyme: To obtained detailed information on the composition of a particular group of intestinal microbes that have be associated with obesity in previous studies, a total of 16 genera were quantified by real-time PCR with group-specific primers. The results are shown in Table 3-5. The percent of Bifidobacterium in the colons of LCBs were higher than those in LD. Significantly higher Bifidobacterium percent were also found in the ileum of BM. The ileum of LCBs contains significantly less *Staphylococcus aureus* in ileum than that of LD. *Escherichia* was more abundance in the ileum of LCBs than that of LD. LCBs except for NX, contain less Lactobacillus than LD. LCBs, except for NX have a greater content of Prevotella than LD. LCBs, except for NX have a greater content of *Faecalibacterium prausnitzii* than LD. The percent of *Clostridium perfringens* in LCBs were higher in ileum and cecum there was a lower content in colon. While NX, LT and HJ show this trend for a decrease in *Methanobrevibacter smithii*, Bama mini-pig shows an opposite tendency.

In this study, researchers found that the ratio of Bacteriodetes/Firmicutes in the colon in LCBs was significantly lower than that in LD. However, there was no similar difference in the other two intestinal segments

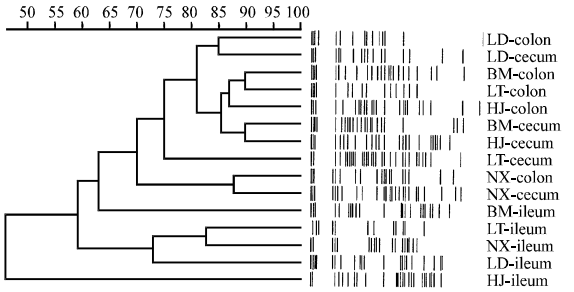

Fig. 1: V3 region of *16S rRNA* gene profile from contents sampled from three intestine segments (ileum, cecum and colon) sampled from four LCBs (HJ, BM, NX and LT) and LD analyzed by DGGE. Dendrogram of DGGE profiles which were analyzed using UPGMA Method

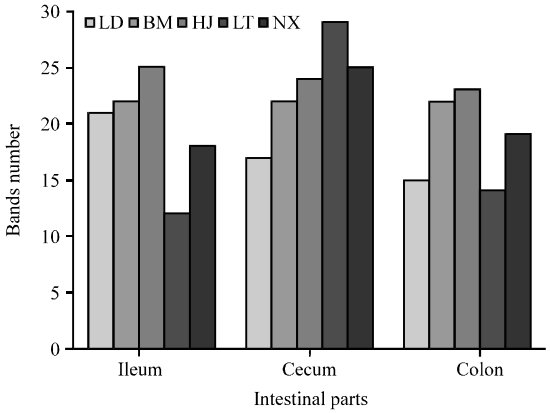

Fig. 2: Bands number of the DGGE analysis on intestinal microbiota composition of LCBs and LD

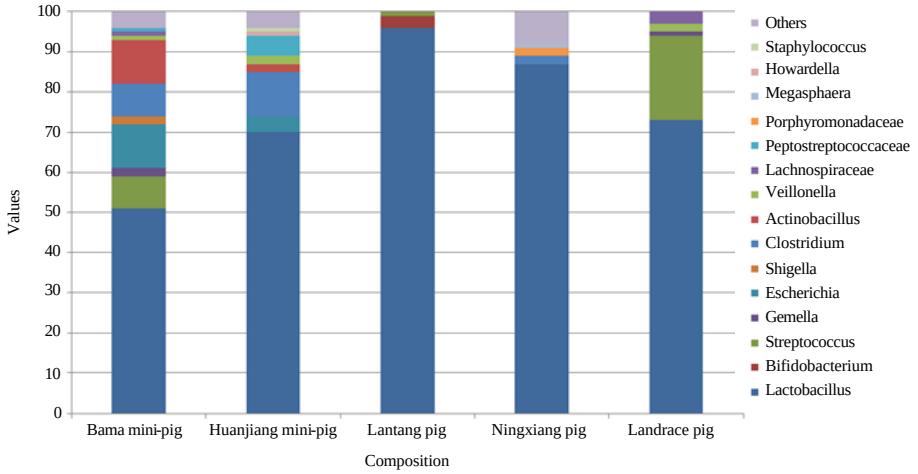

Fig. 3: Genus-level composition of ileal microbiota in four LCBs and LD

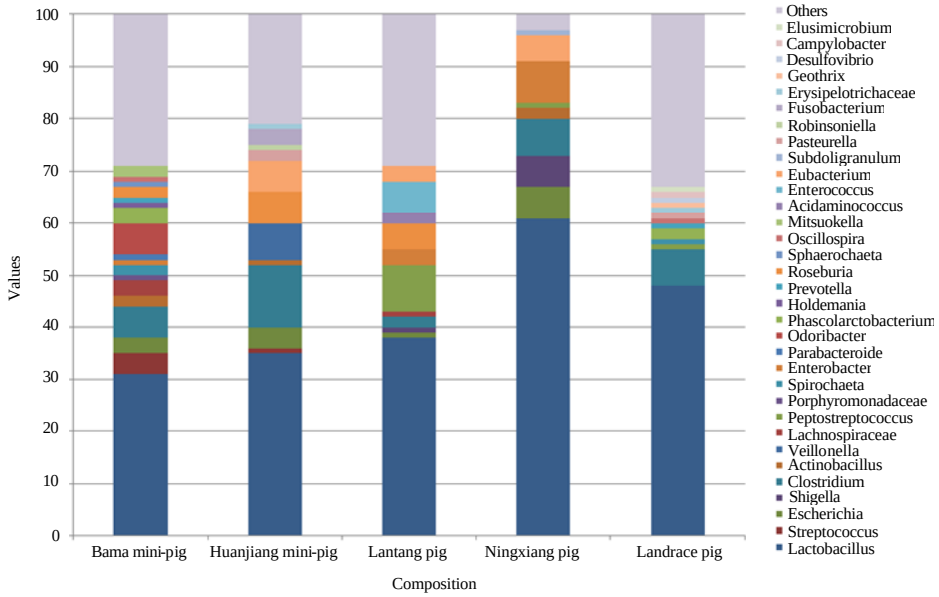

Fig. 4: Genus-level composition of cecal microbiota in four LCBs and LD

Table 3: Bacterial populations measured by qPCR in ileac contents sampled from four LCBs and LD (n = 12)

Bacterias	BM	LT	NX	HJ	LD
Bifidobacterium	2.61±0.22^{a}	0.99±0.03^{b}	0.06±0.01^{c}	0.19±0.01^{c}	1.14±0.08^{b}
Lactobacillus	0.16±0.01^{d}	0.10±0.02^{b}	1.39±0.08^{a}	0.24±0.02^{d}	0.79±0.05^{c}
Peptostreptococcus	0.71±0.04^{cd}	0.83±0.03^{c}	2.41±0.14^{b}	0.35±0.05^{d}	3.62±0.33^{a}
Escherichia	2.32±0.13^{a}	1.43±0.10^{b}	0.06±0.005^{d}	0.29±0.01^{c}	0.16±0.01^{cd}
Bacteroidetes	1.05±0.09^{ab}	1.03±0.09^{ab}	0.85±0.11^{b}	1.21±0.05^{a}	1.01±0.09^{ab}
Bacteroides thetaiotaomicron	-	2.91±0.36^{b}	0.002±0.0006^{c}	-	5.22±0.72^{a}
Firmicutes	0.27±0.07^{d}	0.76±0.11^{cd}	2.01±0.26^{b}	3.22±0.16^{a}	1.26±0.20^{c}
Staphylococcus aureus	-	0.0003±0.00001^{b}	-	-	4.53±0.19^{a}
Clostridium coccoides group	0.04±0.008^{a}	0.05±0.01^{a}	0.001±0.0001^{c}	0.013±0.002^{bc}	0.03±0.004^{ab}
Clostridium leptum subgroup	3.11±0.60^{a}	2.67±0.17^{a}	0.11±0.02^{b}	0.38±0.06^{b}	0.88±0.07^{b}
Clostridium perfringens group	1.35±0.11^{a}	-	-	0.33±0.03^{b}	0.33±0.06^{b}
Clostridium coccoides-Eubacteria	0.008±0.001^{b}	0.02±0.001^{b}	1.30±0.30^{a}	0.004±0.001^{b}	0.09±0.02^{b}
Prevotella	0.09±0.008^{a}	0.06±0.007^{a}	0.002±0.0006^{b}	-	0.01±0.01^{b}
Faecalibacterium prausnitzii	0.30±0.02^{b}	-	0.004±0.0007^{b}	0.002±0.0004^{b}	5.00±0.97^{a}
Fusobacterium prausnitzii	2.70±0.37^{a}	-	0.018±0.005^{b}	-	0.0014±0.0002^{b}
Methanobrevi bactersmithii	-	0.13±0.005^{a}	0.15±0.02^{a}	-	0.012±0.002^{b}
Roseburia	-	0.87±0.05^{a}	0.006±0.0004^{b}	-	0.024±0.002^{b}

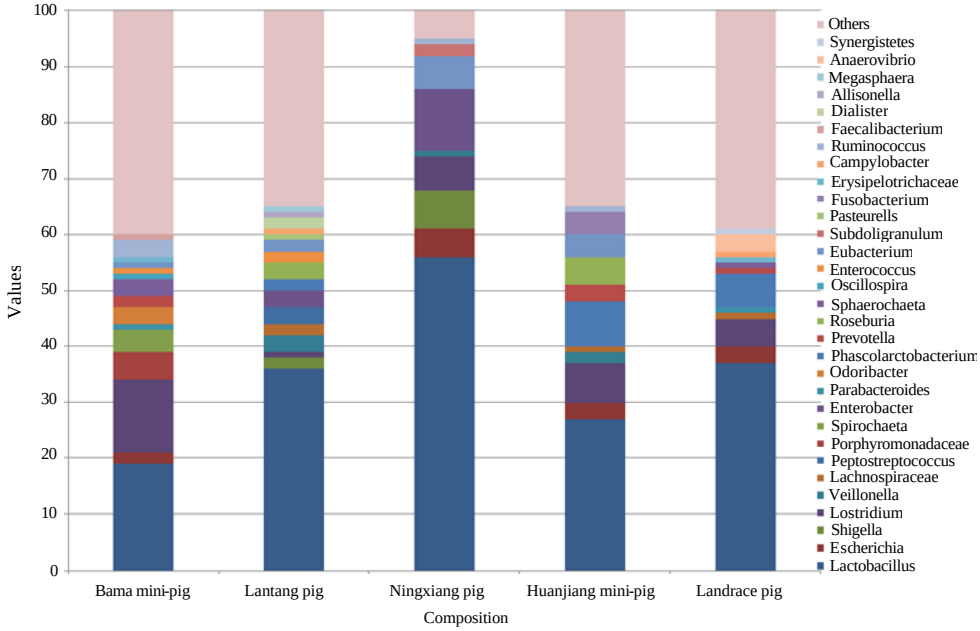

Fig. 5: Genus-level composition of colonic microbiota in four LCBs and LD

Table 4: Bacterial populations measured by qPCR in cecal contents sampled from four LCBs and LD (n = 12)

Bacterias	BM	LT	NX	HJ	LD
Bifidobacterium	0.42±0.01^{bc}	0.14±0.01^{d}	0.60±0.02^{a}	0.39±0.01^{c}	0.45±0.02^{b}
Lactobacillus	0.41±0.01^{d}	0.34±0.01^{e}	1.47±0.04^{a}	0.49±0.03^{c}	0.66±0.02^{b}
Peptostreptococcus	0.33±0.02^{b}	0.24±0.02^{c}	0.79±0.03^{a}	0.37±0.01^{b}	0.12±0.01^{d}
Escherichia	0.26±0.01^{c}	0.35±0.03^{b}	1.48±0.03^{a}	0.32±0.005^{b}	0.32±0.006^{b}
Bacteroidetes	1.66±0.07^{a}	1.78±0.06^{a}	0.99±0.10^{b}	1.15±0.14^{b}	1.09±0.08^{b}
Bacteroides thetaiotaomicron	1.08±0.16^{a}	0.42±0.03^{b}	0.13±0.01^{c}	0.08±0.03^{c}	0.11±0.02^{c}
Firmicutes	0.13±0.06^{b}	1.50±0.25^{a}	0.27±0.12^{b}	1.19±0.26^{a}	1.06±0.19^{a}
Staphylococcus aureus	0.009±0.0003^{a}	-	0.006±0.0002^{b}	-	-
Clostridium coccoides group	0.33±0.04^{b}	3.44±0.64^{a}	0.39±0.08^{b}	1.21±0.16^{b}	2.60±0.24^{a}
Clostridium leptum subgroup	0.50±0.04^{b}	0.54±0.06^{b}	2.12±0.25^{a}	0.55±0.07^{b}	0.45±0.14^{b}
Clostridium perfringens group	2.71±0.71^{a}	0.002±0.0003^{b}	0.005±0.0007^{b}	0.005±0.0008^{b}	-
Clostridium coccoides-Eubacteria	0.005±0.001^{b}	0.66±0.07^{a}	0.01±0.0002^{b}	0.09±0.005^{b}	0.008±0.0007^{b}
Prevotella	1.77±0.09^{a}	1.52±0.19^{a}	0.08±0.01^{c}	0.57±0.08^{b}	0.46±0.04^{b}
Faecalibacterium prausnitzii	2.00±0.27^{a}	0.60±0.10^{b}	0.08±0.01^{c}	2.17±0.17^{a}	0.86±0.13^{b}
Fusobacterium prausnitzii	0.43±0.07^{c}	0.42±0.05^{c}	0.08±0.01^{c}	3.71±0.09^{a}	2.17±0.29^{b}
Methanobrevi bactersmithii	1.08±0.08^{a}	0.43±0.06^{c}	0.86±0.08^{b}	0.38±0.03^{c}	0.68±0.08^{b}
Roseburia	-	0.015±0.003^{b}	0.039±0.002^{a}	0.058±0.009^{a}	-

Table 5: Bacterial populations measured by qPCR in colonic contents sampled from four LCBs and LD (n = 12)

Bacterias	BM	LT	NX	HJ	LD
Bifidobacterium	0.68±0.05^{b}	0.25±0.01^{c}	3.22±0.11^{a}	0.88±0.03^{b}	0.42±0.015^{c}
Lactobacillus	0.21±0.01^{c}	0.18±0.01^{c}	1.33±0.09^{a}	0.30±0.01c	0.70±0.008^{b}
Peptostreptococcus	0.07±0.003^{c}	0.13±0.003^{bc}	1.17±0.06^{a}	0.22±0.02^{b}	0.16±0.002^{bc}
Escherichia	0.30±0.01^{c}	0.41±0.002^{bc}	2.55±0.09^{a}	0.52±0.01^{b}	0.47±0.004^{b}
Bacteroidetes	0.06±0.01^{c}	0.56±0.16^{b}	1.30±0.12^{a}	0.71±0.07^{b}	1.33±0.005^{a}
Bacteroides thetaiotaomicron	0.21±0.04^{b}	1.34±0.12^{a}	0.29±0.07^{b}	0.03±0.006^{b}	0.05±0.006^{b}
Firmicutes	0.15±0.07^{a}	0.28±0.14^{a}	0.36±0.13^{a}	0.49±0.24^{a}	0.05±0.004^{a}
Staphylococcus aureus	0.01±0.0003^{b}	5.51±0.40^{a}	-	0.02±0.003^{b}	0.01±0.001^{b}
Clostridium coccoidesgroup	2.85±0.31^{a}	1.75±0.33^{b}	0.18±0.02^{c}	0.78±0.09^{c}	0.12±0.002^{c}
Clostridium leptumsubgroup	1.15±0.12^{a}	0.20±0.06^{c}	0.97±0.05^{a}	0.33±0.006^{c}	0.65±0.004^{b}
Clostridium perfringensgroup	0.002±0.001^{b}	0.0005±0.00009^{b}	-	0.008±0.0006^{b}	3.00±0.600^{a}
Clostridium coccoides-Eubacteria	0.10±0.01^{b}	0.17±0.02^{a}	0.003±.0006^{c}	0.037±0.006^{c}	0.01±0.001^{c}
Prevotella	4.49±0.67^{a}	0.89±0.07^{b}	0.10±0.01^{b}	1.14±0.16^{b}	0.36±0.005^{b}
Faecalibacterium prausnitzii	1.18±0.09^{a}	0.97±0.12^{a}	0.17±0.04^{c}	0.56±0.04^{b}	0.18±0.006^{c}
Fusobacterium prausnitzii	0.29±0.04^{c}	1.19±0.07^{a}	0.07±0.01^{d}	0.88±0.004^{b}	0.35±0.009^{c}
Methanobrevibacter smithii	3.42±0.52^{a}	0.66±0.04^{b}	0.99±0.12^{b}	0.46±0.06^{b}	2.87±0.042^{a}
Roseburia	0.26±0.01^{c}	0.91±0.03^{c}	5.83±0.40^{a}	2.19±0.07^{b}	-

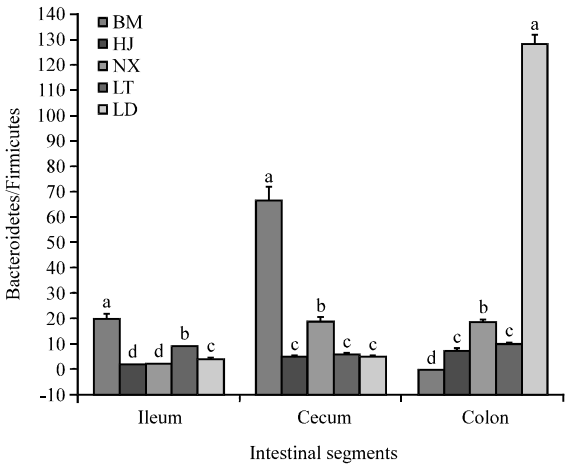

Fig. 6: The ratio of Bacteriodetes/Firmicutes in content of three intestinal segments in LCBs and LD

(ileum and cecum) and in fact the Bacteriodetes/Firmicutes ratios in intestinal microbiota in HJ were significantly higher than that in LD (Fig. 6).

DISCUSSION

The primary reason of obesity is excess energy intake, even <1% compared with daily expenditure can lead to a detrimental increase in body weight in the long term (Hill, 2006). One of the mechanisms that could underlie such an excess is an increase in energy extraction from indigestible food components. Intestinal microbiota contribute this process by influencing the efficiency of calorie harvest from the diet and how harvested energy is used and stored (Backhed *et al*., 2004; Samuel *et al*., 2008). For the difference of nutrition contents of diet and various raising environments, the nutrition contents of sow milk of LCBs and LD also are different (Zou *et al*., 1992) and these will influence the infant intestinal microbiota composition and then further induce the clear differences in the genotype and phenotype among LCBs and LD together with other factors after weaning (He *et al*., 2012). That is because the infant intestinal microbia composition could predict the body weight when they grow up (Kalliomaki *et al*., 2008). In this study, researchers analyzed and compared the composition of intestinal microbiota in LCBs and LD. Several studies have highlighted a correlation between the structure of the intestinal microbial community and obesity (Nadal *et al*., 2009). Obesity has been associated with a significant decrease in the level of microbiota diversity. But in the study, results shows the microbiota composition in the distal intestine is more complex in LCBs than in LD which appears to challenge this association.

Previous studies have shown that the percentage body fat is correlated with the abundance of bacteroidetes and firmicutes in the intestinal microbiota (Turnbaugh *et al*., 2006). Obese mice and humans have a decrease in bacteriodetes and an increase in firmicutes (Duncan *et al*., 2008). In this study, researchers found that the ratio of bacteriodetes/Firmicutes in the colon in LCBs was significantly lower than that in LD. However, there was no similar difference in the other two intestinal segments (ileum and cecum) and in fact the bacteriodetes/firmicutes ratios in intestinal microbiota in HJ were significantly higher than that in LD. These result is different previous study that obese pigs from the Banna mini-pig inbred line had lower percentages of Bacteroidetes but a similar percentage of Firmicutes in their feces, compared to the lean pigs from same line (Guo *et al*., 2008a, b). In this study, the LCBs studied had lower lean meat ratios than LD (62%): BM (59.36%), HJ (50%, Wu *et al*., 2012), NX (38.87%, Hu *et al*., 2011) and LT (33.3%). The lean meat ratio and the bacteriodetes/ firmicutes in ratio in the colon seems to be positively correlated.

The intestinal microbiota composition at the species level is related to body weight and obesity which might be important for further studies and the management of obesity. Previous studies have found significant complex associations between obesity and an increase in some bacterial groups (Pennisi, 2011) including Lactobacillus, *Staphylococcus aureus*, *Escherichia coli*, *Faecalibacterium prausnitzii*, etc. Conversely, other groups mainly belonging to the Bifidobacterium genus have been associated with a lean status (Collado *et al*., 2008). In present study, researchers quantified some genera that have been shown to be associated with obesity. High Bifidobacterium counts are especially important (Chierici *et al*., 2003) due to their metabolic activities which beneficially influence the host. Bifidobacterium can influence the precursor pool for serotonin which is related to food intake (Desbonnet *et al*., 2008). In obese infancy, bifidobacterial numbers in fecal samples were lower (Kalliomaki *et al*., 2008). When mice with high-fat diet-induced obesity were supplied a diet containing *Bifidobacter breve* they showed a significant weight decrease (Kondo *et al*., 2010). In this study, the Bifidobacterium counts in the colons of LCBs were higher than those in LD. Significantly higher Bifidobacterium counts of were also found in the ileum of BM. These results were not consistent with previous findings that obese LCBs have more symbiotic Bifidobacterium. A possible explanation for this discrepancy is that bifidobacteria are just one factor and they do not make a crucial contribution to obesity. More significant contribution of Bifidobacterium may be that they reduce intestinal endotoxin levels and improve

mucosal barrier function (Griffiths *et al*., 2004), inhibit intestinal colonization by pathogenic microorganisms and have immunostimulatory and anti-diarrheal properties (Russell *et al*., 2011). The fact that the ileum of LCBs contains significantly less *Staphylococcus aureus* in ileum than that of LD which is consistent with the earlier fact. *Staphylococcus aureus* is one of the most significant pathogens and causes both nosocomial and community-acquired infections. The microbiota aberrance during infancy in children who become overweight has also been associated with a greater number of *Staphylococcus aureus* (Kalliomaki *et al*., 2008). *Staphylococcus aureus* may indeed act as a trigger of low-grade inflammation (Lundell *et al*., 2007) and contribute to the development of obesity (Kalliomaki *et al*., 2008). Interestingly, there seems to be a greater abundance of Escherichia in the ileum of LCBs than in that of LD. Lactobacillus can produce bile salt hydrolases that deconjugate bile salt complexes and reduce lipid solubilization and absorption and even lower cholesterol levels (Begley *et al*., 2006) and be able to reduce abdominal adiposity and body weight (Luoto *et al*., 2010). In general, LCBs except for NX, contain less Lactobacillus than LD. This result may be related to the composition of Lactobacillus. While most of Lactobacillus can reduce fat accumulation some have been shown to significantly increase fat deposition (Lu *et al*., 2010). To clarify the association between Lactobacillus contents and phenotypes of LCBs, more studies are needed with a particular focus on the Lactobacillus.

Intestinal bacteria mostly use fermentation to generate energy in which they convert sugars in part to Short-Chain Fatty Acids (SCFAs) that are used by the host as an energy source (Yin *et al*., 2000). SCFA resulting from colonic fermentation may provide an estimated additional 10% daily dietary energy to the host which may be used for *de novo* hepatic triglyceride and glucose synthesis (Flint *et al*., 2008; Scheppach, 1994). As such, a mere increase of 1% in metabolic activity could provide an additional 20 kcal day^{-1} based on a 2,000 kcal day^{-1} diet, resulting in an annual weight gain of nearly 1 kg. SCFA mainly contains acetate, propionate butyrate, lactate and formate. Acetate is important for muscle, heart and brain cells (Wong *et al*., 2006) and propionate is used in host hepatic neoglucogenic processes. Members of the Prevotella group play important roles in the hydrolysis and fermentation of dietary fiber and produce acetate and propionate (Salyers, 1984). The present results showed that LCBs, except for NX have a greater content of Prevotella than LD. Butyrate, a major component of the energy cycle is important for enterocytes (Hamer *et al*., 2008) and is produced by several bacteria including *E. rectale-C. coccoides* group and *Faecalibacterium prausnitzii* (Louis and Flint, 2009). Higher butyrate and propionate concentrations have been identified in obese individuals (Schwiertz *et al*., 2010). LCBs, except for NX, have a greater content of *Faecalibacterium prausnitzii* than LD. The results showed that LCBs were more strongly influenced by intestinal microbiota, since *Faecalibacterium prausnitzii* has been identified as one of the key functional members of the microbiome that most strongly influences the host metabolism (Li *et al*., 2008) and is responsible for a significant proportion of the fermentation of unabsorbed carbohydrate. An increase in the mumber of *Faecalibacterium prausnitzii* in the feces of obese children in South India adds to the growing body of information on changes in the fecal microbiota in obesity (Balamurugan *et al*., 2010) and this finding is consistent with the result. The results support the notion that obese LCBs may generate more SCFA by fermentation by intestinal microbiota. Intestinal microbiota show different metabolic and compositional adaptations in response to substrate availability. A high energy medium is strongly butyrogenic and results in the significant stimulation of butyrate-producing members of clostridia cluster XIVa whereas members of cluster IV demonstrate greater adaptive variability. Normal and low-energy nutrient loads induce significantly less metabolic activity in both microbiota and low-energy medium induce a broad reorganization of the commensal community structure (Payne *et al*., 2012). Although, the *Clostridium perfringens* counts in LCBs were higher in ileum and cecum there was a lower counts in the colon which is consistent with that the finding that an obese group showed a lower amount of *Clostridium perfringens* than a normal-weight group (Zuo *et al*., 2011). *Bacteroides thetaiotaomicron* is also a prominent member of the human distal intestinal microbiota and has an extraordinary capacity for acquiring and degrading plant polysaccharides (Xu *et al*., 2003). Its proteome contains 172 glycosyl hydrolases that are predicted to cleave most glycosidic linkages encountered in the diet. LCBs are mainly fed roughage. *Bacteroides thetaiotaomicron* is highly efficient at glycan metabolism which enables otherwise indigestible sugars to be metabolized and harvested as additional energy (Comstock and Coyne, 2003). LCBs had higher counts of *Bacteroide sthetaiotaomicron* promote the ability to harvest more energy through the induction of host monosaccharide transporters (Hooper *et al*., 2001). *Methanobrevibacter smithii*, a hydrogen-consuming

methanogen, is the most prominent archaea in humans and comprises 10% of all anaerobes (Eckburg *et al*., 2005). Co-colonization by *Methanobrevibacter smithii* and *Bacteroides thetaiotaomicron* increased the efficiency of energy extraction from dietary polysaccharides and the amount of host adiposity more than colonization with either organism alone (Samuel and Gordon, 2006). Furthermore, *Methanobrevibacter smithii* influenced the metabolism of *Bacteroides thetaiotaomicron* and prompted it to consume mainly fructose-containing polysaccharides that break down into several substances including formate an important source of energy for *Methanobrevibacter smithii* (Samuel *et al*., 2007; Zhou *et al*., 2012). Obesity has been associated with a specific profile of the bacterial intestinal microbiota including a significant decrease in *Methanobrevibacter smithii*, the leading representative of the intestinal microbiota archaea (Million *et al*., 2012). While NX, LT and HJ show this trend for a decrease in *Methanobrevibacter smithii*, Bama mini-pig shows an opposite tendency. *Archaeal methanogenesis* improves the efficiency of polysaccharide fermentation by preventing the production of hydrogen and other reaction end-products. By removing hydrogen and formate, *Methanobrevibacter* species may help the bacterial community produce more acetate and butyrate which are important carbon sources for colon epithelium cells.

CONCLUSION

Researchers compared the intestinal microbiota compositions between LCBs and LD. The results indicated that the microbiota diversity in the distal intestine in LCBs was significant higher than in LD which challenge the result that the microbiota diversity decrease in obese individuals. Intestinal microbiota do contribute for the obese phenotype but genotype is the main contributor. Furthermore, the ratio of Bacteriodetes/Firmicutes in the colon is significantly lower in LCBs than in LD which shows a positive influence as in previous reports, other genera that influence the energy balance showed complex content patterns. Researchers confirmed the association between the intestinal microbiota compositions with infancy later obesity in pigs. Each pig breed was also found to have a unique microbiota composition. While BM and HJ seems to have similar microbiota compositions, NX is distinct among LCBs. The higher contents of some intestinal microbial populations that have strong energy-harvesting ability may partly contribute to the obese phenotype of LCBs. Researchers also confirmed LCBs can be used as animal model to do research on obesity but more studies on LCBs should be explained on the contribution of the microbiota composition in infancy to adult obesity.

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