https://doi.org/10.17221/225/2020-CJAS

Analysis of the relationship between caecal flora difference and production performance of two rabbit species by high-throughput sequencing

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Citation: Guo Z.Q., Wang B., Lu J.Z., Li C.Y., Kuang L.D., Tang X.X., Mei X.L., Xie X.H. (2021): Analysis of the relationship between caecal flora difference and production performance of two rabbit species by high-throughput sequencing. Czech J. Anim. Sci., 66: 271–280.

Abstract: The purpose of this experiment is to study the relationship between the difference in production performance between Sichuan White (SC) rabbits and New Zealand (NZL) rabbits and the diversity of caecal flora. Twelve pregnant SC rabbits and 12 NZL female rabbits were selected for this experiment. After delivery, the young rabbits were divided into two groups according to breeds, each group had 30 replicates, and each replicate had one rabbit. During the experiments, these rabbits were kept in the same room, and the temperature in the room was controlled at 12–25 °C, with a 16-hour light cycle every 24 hours. The nutritional composition of the feed and other environmental conditions were consistent. On the 59th day of the experiment, the caecum contents of the two groups of young rabbits were collected. The results showed that the survival rate of the SC rabbit group was higher than that of the NZL rabbit group, and the diarrhoea rate and average daily gain were lower than those of the NZL rabbit group ($P < 0.05$). The results of high-throughput sequencing of the 16S gene showed that compared with the NZL rabbit group, the relative abundance of *Bacteroides* increased, and the abundance of harmful flora *Verrucomicrobia* and *Proteobacteria* decreased ($P < 0.05$). Functional analysis of the microflora showed that the relative abundance of carbohydrate metabolism genes in the SC rabbit group was higher than in the NZL rabbit group. In conclusion, compared with the NZL rabbits, the SC rabbits have a more optimized intestinal flora structure and lower abundance of harmful bacteria. Moreover, the intestinal health level of SC rabbits is improved, and the tolerance to roughage of SC rabbits is increased.

Keywords: comparative study; gut microflora; high-throughput sequence; New Zealand rabbit; Sichuan White rabbit

The Sichuan White Rabbit (SC) is one of the only two Chinese domestic rabbit breeds in the "World Breeds and Their Breeding History" with genetic characteristics such as high reproductive performance, wide adaptability, tender meat and small size. Besides, The SC Rabbit gets excellent performance in resistance to adversity such as rough feeding, blood mating and heat tolerance (Yang et al. 2019).

In recent years, many studies have shown that the animal caecal microflora plays an important role

Supported by the National Rabbit Industry Technology System of China (No. CARS-43-D-3), Sichuan Provincial Scientific Research Institute and Technological Achievements Transformation Fund Project (No. 20YSZH0013).

https://doi.org/10.17221/225/2020-CJAS

in the host's nutrient digestion, absorption, and gut health. For example, the caecal microflora can degrade cellulose, hemicellulose, and insoluble proteins. At the same time, it is important for the development of the maturation of terminal immune organs (Guarner and Malagelada 2003; Skrivanova et al. 2008; Quast et al. 2013). Establishing a healthy, stable and diverse intestinal flora structure in the digestive tract helps to prevent intestinal diseases, especially in stressful periods such as weaning (Frese et al. 2015). Spor et al. (2011) demonstrated a significant correlation between the genotype of the host and the composition of gut microflora. The research conducted by Fan et al. (2020) showed that although cattle with different genetic backgrounds were raised in the same environment, the microbial composition of the caecum was quite different. This finding indicates that the composition of the caecum lumen microbes is related quantitatively and structurally to the host itself.

In this experiment, the SC rabbits and the New Zealand (NZL) rabbits were subjected to the comparative study of their caecum flora by using the 16S rRNA gene Miseq high-throughput sequencing technology. This aims to analyse the intestinal core flora, further illustrating the relationship between the composition of the caecum flora of the two rabbit breeds and the differences in their respective production performance, which is important for analysing the digestive and physiological mechanisms of SC rabbits, further breeding and developing feeding standards.

Table 1. Ingredients and proximate analysis of nutrients in basal ration

Feed ingredients	Female rabbit feed	Young rabbit feed
Feed composition (%)		
Alfalfa meal	30.00	24.00
Soybean meal (CP 43%)	14.00	10.00
Soybean oil	0.50	–
Expanded soybean	–	3.00
Corn	16.00	15.00
Rice husk	5.00	11.00
Full fat rice bran	10.00	8.00
Rapeseed meal	5.30	5.50
Wheat bran	15.70	20.00
L-Lys·HCl (98%)	0.10	0.10
NaCl	0.50	0.50
$CaHPO_4$	1.20	1.20
$CaCO_3$	0.70	–
Limestone	–	0.70
Premix[1]	1.00	1.00
Total	100.00	100.00
Nutrient levels[2]		
Digestible energy (MJ/kg)	11.20	10.50
Crude fibre (%)	14.23	15.95
Crude protein (%)	17.52	15.64
Ca (%)	1.03	1.07
Total protein (%)	0.67	0.65

[1]The premix provides the following per kg of diet: Fe 100 mg, Cu 20 mg, Zn 90 mg, Mn 30 mg, Mg 150 mg, vitamin A 4 000 IU, vitamin D_3 1 000 IU, vitamin E 50 mg and choline 1 mg

[2]Digestabe energy was calculated value, while other nutrient levels were measured values

MATERIAL AND METHODS

The experiment was carried out at the Animal Science and Technology farm of Southwest University, and all study procedures were approved by the Southwest University Animal Care and Use Committee (IACUC No.20191015-02) (Chongqing, China).

Experimental animals and sample collection

Twelve healthy SC rabbits and twelve NZL female rabbits, each weighing about 4.5 kg, were selected for the experiment in their third litter and fed the compound diet for females (Table 1) in a single cage during the experiment. Young rabbits of the two breeds of female rabbits were collected after birth and each young rabbit was marked according to the breed, which was fostered to other female rabbits delivered at the same time. The purpose was to ensure that the intestinal microflora of the experimental young rabbits was not disturbed while there is a different breed of female rabbits. The young rabbits were weaned uniformly at 28 days of age and fed a basal diet (Table 1) after weaning and kept in a single cage. Before the test, the rabbit house and cage were cleaned and disinfected. During the experiment, the ambient temperature was controlled at 12–25 °C, and the light cycle was 16 hours.

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Besides, the feeding environment as well as the diet were the same, and all rabbits could feed and drink autonomously. Immunization was given following the routine procedure, and the rabbit house provided rabbits with natural light and ventilation, while they were fed at 08:00 and 17:00.

Production performance measurement

The experiment lasted for 59 days, including a 7-day period before a female rabbit gives birth, a 28-day breastfeeding period and a 14-day fattening period. The rabbits were weighed individually once a week during the experiment, and the average daily gain was calculated. The numbers of diarrhoea and mortality of rabbits were recorded every day by the same persons who administered the diets. The incidence of diarrhoea was calculated as follows: diarrhoea incidence (%) = [(number of rabbits with diarrhoea)/(number of experimental rabbits × experimental days)] × 100%, where the number of rabbits with diarrhoea (clinical signs of the disease including transitory diarrhoea, presence of mucus in excreta, etc.) was the summation of the number of rabbits with diarrhoea every day with all rabbits in each group. The incidence of mortality was calculated as follows: mortality incidence (%) = (number of rabbits with mortality per group during the trial/total number of test rabbits per group) × 100% (Wang et al. 2017).

Sample collection and preservation

On day 59 of the experiment, 12 young rabbits (weighing 755 ± 26 g) were randomly selected from each breed. The rabbits were electrically stunned and exsanguinated via the carotid arteries and jugular veins. After the caecum was ligated, the ligated intestine together with the contents was packed into 20-ml sterile ultra-low temperature storage tubes and then transferred to –80 °C for storage.

Sample DNA extraction and PCR amplification

The total bacterial DNA of caecum contents was extracted using a faecal genomic DNA extraction kit. The integrity of the extracted genomic DNA was detected with 1.5% agarose gel electrophoresis, and the concentration of DNA and optical density (OD)260/OD280 values were measured by an ultraviolet spectrophotometer. The samples that passed the test were entrusted to Shanghai Pessenno Biological Detection Company for PCR amplification of the V3-V4 region of the bacterial 16S rRNA gene, and the PCR products were quantified and then sequenced by Illumina HiSeq 2500 sequencing platform for high-throughput sequencing according to the sequencing volume requirement of each sample.

Data analysis

The paired-end (PE) base sequence we obtained from MiSeq sequencing was firstly spliced according to overlap relationships. After the sequence was quality-controlled, filtered, and de-hybridized, the samples were differentiated. The obtained sequences were subjected to operational taxonomic unit (OTU) clustering analysis, and a clustering program (v1.2.22) was used for species taxonomic analysis based on a 97% sequence identification rate of the SILVA database (Max Planck Institute for Marine Microbiology and Jacobs University, Bremen, Germany). That taxonomic information on the species corresponding to each OTU was obtained by comparing the 16S bacterial and archaeal ribosome database Greengenes (Release 13.8, http://greengenes.secondgenome.com). The naive Bayes algorithm was used to perform taxonomic analysis of the OTU representative sequences at a 97% similarity level, and statistics were obtained on the proportion of each sample community at the domain, kingdom, phylum, class, order, family, genus and species taxonomic level, respectively. These 16S rRNA gene amplicon sequences were subsequently subjected to species alpha diversity analysis, species composition analysis, species difference analysis, and phylogenetic investigation of communities by reconstruction of unobserved states (PICRUSt) function prediction (Langille et al. 2013; Fu et al. 2016). All data analyses for growth performance and gut microflora were performed using one-way analysis with SPSS for Windows, v14.0 (SPSS Inc., Chicago, IL, USA), and significant differences between means were compared by one-way ANOVA procedure. The differences were judged as statistically sig-

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nificant at P-values < 0.05, and the analysis result was noted with mean ± standard deviation (M ± SD).

RESULTS

Differences in production performance of rabbit litters

After the weighing selection, the weights of the selected experimental young rabbits are shown in Table 2. After the nursery stage, the results indicated that the SC rabbit group was lower than the NZL rabbit group in terms of average daily weight gain and diarrhoea rate (P < 0.05), while the SC rabbit group was higher than the NZL rabbit group in terms of survival rate (P < 0.05).

Sequencing data statistics

We obtained 927 734 high-quality sequences from 24 samples, with an average of 38 655 ± 3 981.85 sequences per sample. Twelve samples of the SC rabbit group obtained 454 880 valid sequences with an average of 37 906 sequences per sample, and 12 samples of the NZL rabbit group received a total of 472 854 valid sequences with an average of 39 404 sequences per sample (Table 3). According to the OTU division and taxonomic status identification results, the number of microbial taxa contained in the two species at each taxonomic level is shown in Figure 1. The number of microorganisms in the SC rabbit group belonged to 12 phyla, 17 classes, 19 orders, 30 families, 98 genera and four species; the number of microorganisms in the NZL rabbit group belonged to 11 phyla, 16 classes, 19 orders, 29 families, 92 genera and three species. The Venn diagram showed (Figure 2) that based on 97% sequence homology division, the OTUs containing chloroplast were filtered out, and finally 8 498 OTUs were obtained, of which 6 451 OTUs were in the SC group and 6 507 OTUs in the NZL rabbit group. Although the two groups of rabbit breeds were different, they have 4 460 OTUs that are co-owned, accounting for 68.54% and 69.14% of the SC and NZL rabbit groups, respectively. The number of OTUs specific to the SC rab-

Table 2. Production performance data of different breeds of young rabbits

Items	Sichuan White group	New Zealand group	P-value
35-day weight (g)	460.78 ± 4.85[a]	735.67 ± 6.42[b]	< 0.001
Average daily gain (g)	21.43 ± 3.54[a]	32.91 ± 2.85[b]	< 0.001
Diarrhoea rate (%)	12.02 ± 1.84[a]	34.10 ± 4.32[b]	< 0.001
Survival rate (%)	96.60 ± 6.87	92.01 ± 6.92	0.133

Data are presented as means of 12 replications per treatment ± SD

[a,b]Within the row, different lowercase letter superscripts mean significant differences (P < 0.05)

Table 3. Alpha diversity indices of gut microbiome between Sichuan White rabbit and New Zealand rabbit

Items	Sichuan group	New Zealand group	P-value
Vaild sequences	454 880 ± 5 094	472 854 ± 1 820	0.37
Chao1 index	21 032.4 ± 243.55	21 827.6 ± 198.46	0.49
Shannon index	101.7 ± 0.31	98.5 ± 0.40	0.09
Simpson index	11.771 8 ± 0.01	11.8 ± 0.01	0.85

Data are presented as means of 12 replications per treatment ± SD

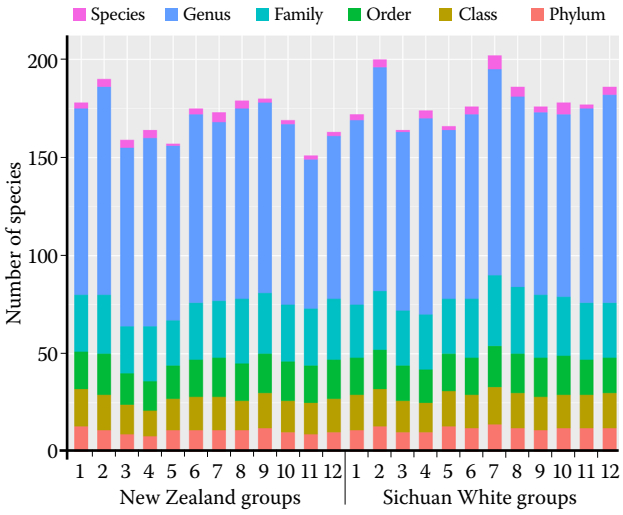

Figure 1. Microbiological groups at various taxonomic levels

Different colours represent different levels of classification

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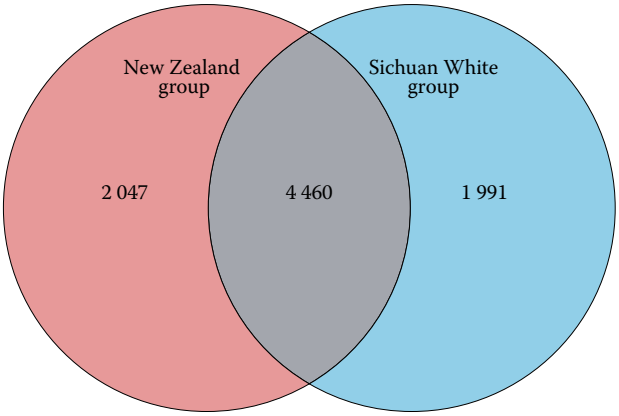

Figure 2. The Venn diagrams show the numbers of functional groups (97% sequence identity) that were shared/not shared by Sichuan White and New Zealand rabbits
Each ellipse represents (a group of) samples, the overlapping area between ellipses indicates the shared operational taxonomic units (OTUs) among samples (groups), and the number of each block shows the number of shared or unique OTUs of the samples (groups) contained in that block

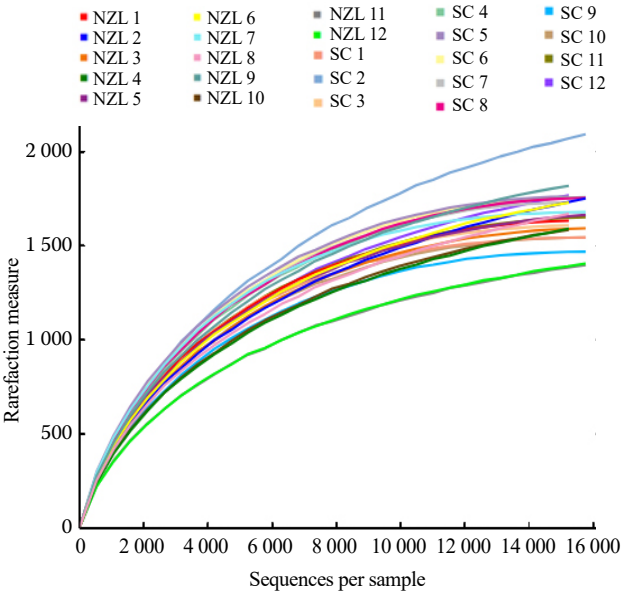

Figure 3. The dilution curve shows that the current sequencing depth can reflect the microbial diversity contained in the community samples
NZL = New Zealand group; SC = Sichuan White group
The longer the curve, the higher the sequencing depth, the higher the possibility of observing higher diversity. The flatness of the curve reflects the influence of the sequenc ing depth on the diversity of the observed samples, and the flatter the curve, the more the sequencing results are suf ficient to reflect the diversity contained in the current samples

bit group (1 991) was lower than that of the NZL rabbit group (2 047).

Alpha diversity analysis

A random sampling method was used to draw dilution curves. As shown in Figure 3, the dilution curves of all samples tended to be flat, indicating that the amount of sequencing data was reasonable and could reflect the information of the majority of microorganisms in the samples. Therefore, the number of OTUs detected in each sample was sufficient for further analysis. The diversity indices of Chao, Shannon and Simpson of caecum microorganisms in the SC rabbit group were not different from those in the NZL rabbit group ($P > 0.05$) (Table 3), indicating that there was no significant difference in caecum flora richness between the two breeds.

Gut flora composition

Differential analysis of bacterially different flora in the sample at the phylum and genus level, based on the abundance of OTU in the sample, and the results are shown in Figures 4 and 5. Table 4 lists the top five bacteria in terms of abundance at the phylum level (SC group: 97.43%; NZL group: 97.66%).

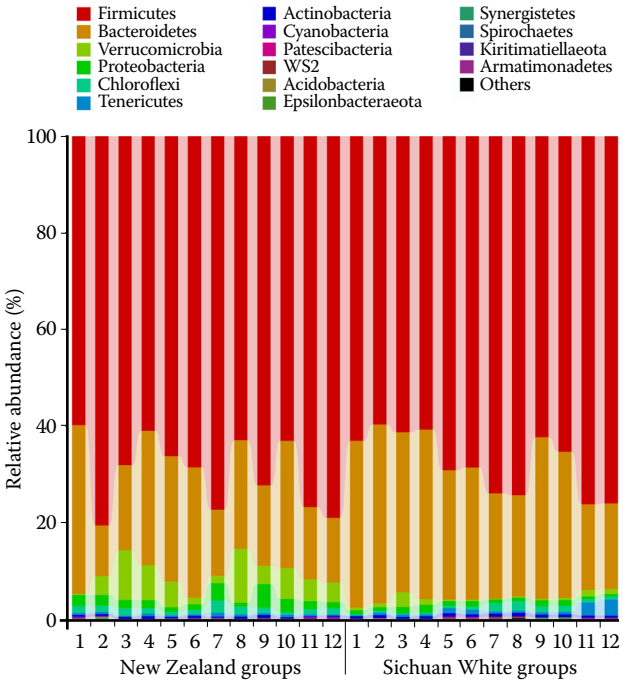

Figure 4. Microbial composition at the phylum level

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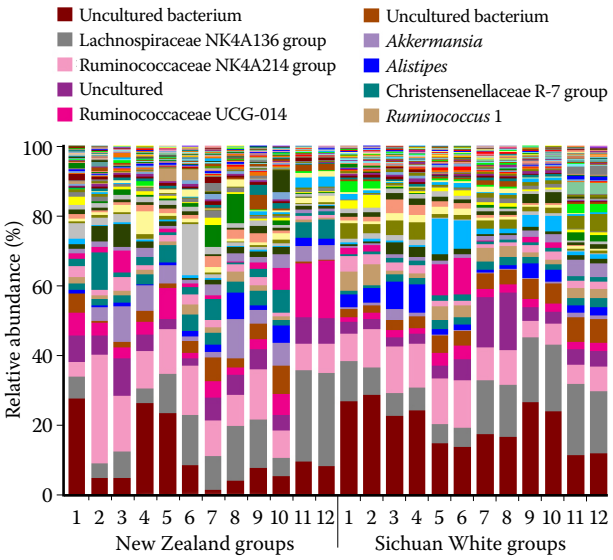

Figure 5. Microbial composition at the genus level

At the phylum level *Firmicutes*, *Bacteroidetes*, *Verrucomicrobia* and *Proteobacteria* were all dominant bacteria in the caecum of the two groups of rabbits, and *Bacteroidetes*, *Verrucomicrobia*, and *Proteobacteria* were the main ones responsible for the differences in the abundance of caecum flora between the two groups of rabbits (Figure 6). As shown in Table 4 at the phylum level, compared to the NZL rabbit group, the SC rabbit group showed an increase of 33.60% and 59.46% in the abundance of *Bacteroidetes* and *Actinobacteria*, respectively ($P < 0.05$), while the abundance of *Verrucomicrobia* and *Proteobacteria* was decreased by 84.24% and 48.57%, respectively ($P < 0.05$).

From Figure 5 it can be seen that the dominant bacteria at the genus level in the caecum stage in both groups of rabbits were uncultured bacteria

Table 4. Relative abundance at the phylum level between Sichuan rabbit and New Zealand rabbit

Species name	Sichuan White-group	New Zealand group	*P*-value
Firmicutes (%)	67.58 ± 6.03	69.66 ± 7.06	0.464 5
Bacteroidetes (%)	27.99 ± 4.38[a]	20.95 ± 4.86[b]	0.001 7
Verrucomicrobia (%)	0.78 ± 0.15[a]	4.95 ± 1.09[b]	0.000 4
Proteobacteria (%)	1.08 ± 0.29[a]	2.10 ± 0.61[b]	0.005 3
Actinobacteria	0.59 ± 0.11[a]	0.37 ± 0.14[b]	0.000 5

Data are presented as means of 12 replications per treatment ± SD

[a,b]Within the row, different lowercase letter superscripts mean significant differences ($P < 0.05$)

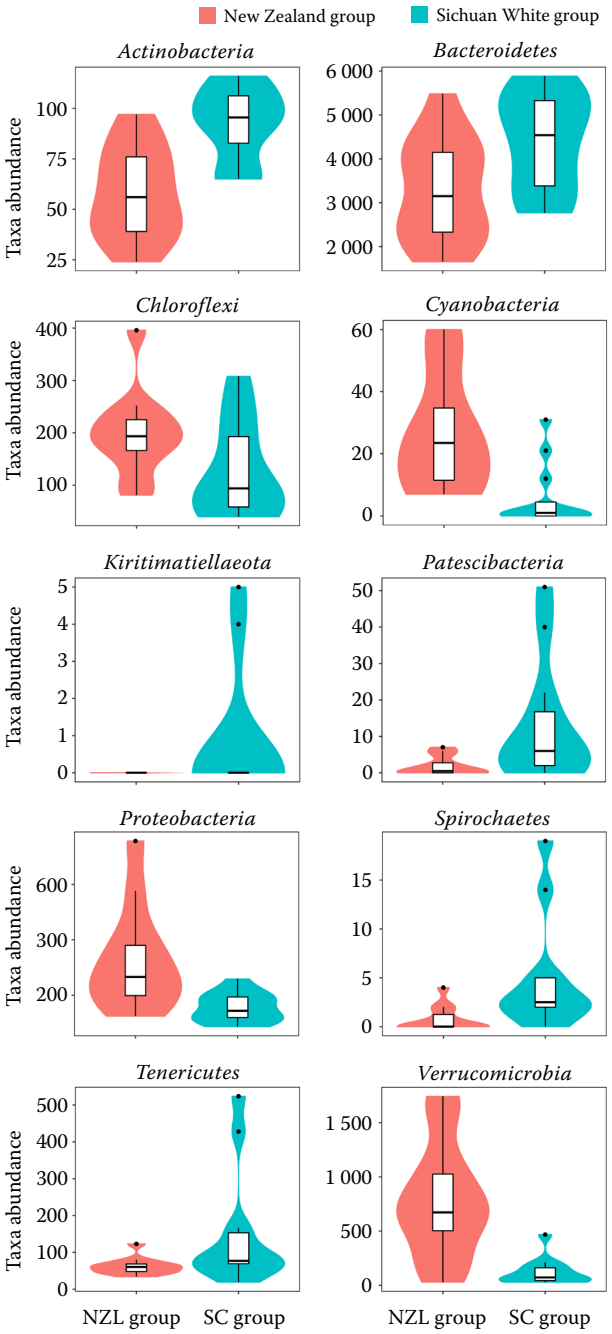

Figure 6. The abundance distribution map of intergroup differences shows the top 10 taxa with the most significant differences

The horizontal coordinates indicate the different groups and the vertical coordinates are the sequence volume of each classification unit within each sample (group). The "fatness" of the violin plot reflects the density of the sample data distribution (the wider the width, the more samples corresponding to the sequence volume)

Lachnospiraceae NK4A136 group, Ruminococcaceae NK4A214 group, Eubacteriaceae; g__uncul-

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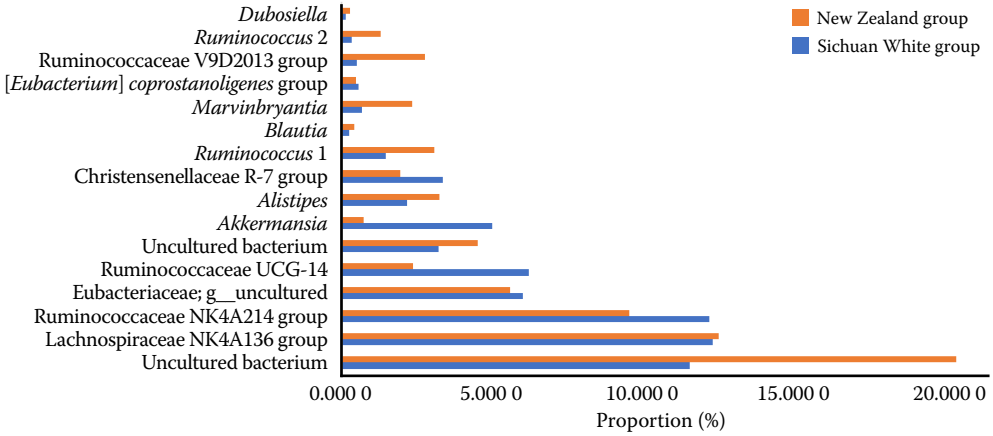

Figure 7. Bacterial community differences at the phylum level
The y-axis represents the species name at the genus level and the x-axis represents relative abundance

tured, Ruminococcaceae UCG-014, uncultured bacterium, *Akkermansia*, *Alistipes*, Christensenellaceae R-7 group and *Ruminococcus* 1. At the genus level, Ruminococcaceae NK4A214 group, Ruminococcaceae UCG-014, *Akkermansia* in the caecum of the SC rabbit group were higher than those of the NZL rabbits group (Figure 7).

Functional analysis of the macro genome

Given the development of data analysis techniques, the metabolic function of microflora can now be predicted on the basis of the sequence data (sequencing results of 16S rRNA genes), thereby matching the "identity" of species with their

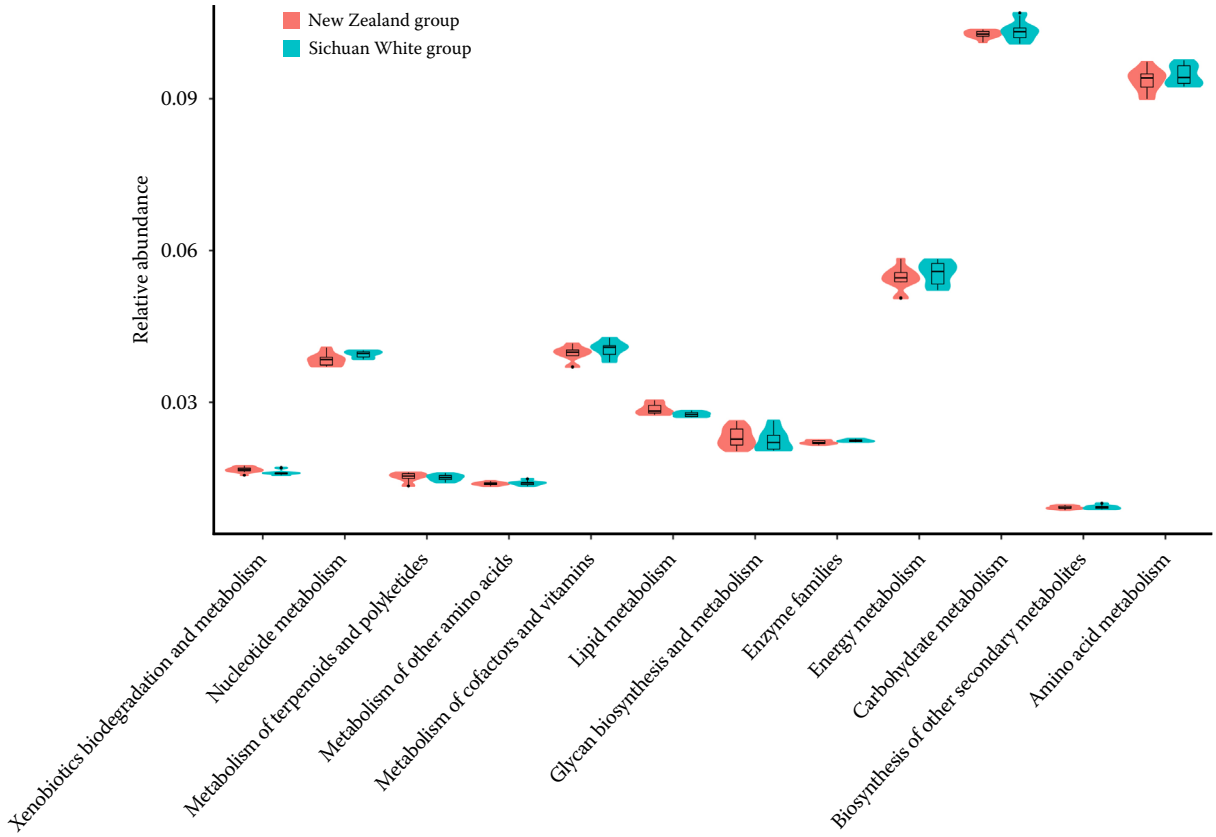

Figure 8. PICRUST predicted the second-level distribution of Kyoto Encyclopedia of Genes and Genomes (www.genome.jp/kegg/) showing the relative abundance of each functional group within each group
The "fatness" of the violin plot reflects the density of the sample data distribution

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"function". The PICRUSt function prediction by applying the method for the functional prediction of caecum microbial genes, 12 functional genes, is shown in Figure 8. The relative abundance of genes from high to low carried out the functions of the metabolism of carbohydrates, amino acids, energy, cofactors, vitamins, nucleotides, lipids, terpenoids and polyketone compounds when the SC rabbits were substantially better than the NZL rabbits in carbohydrate metabolism.

DISCUSSION

Differences in growth performance with relation to diversity and abundance of intestinal flora

We compared the structure of the caecum flora of SC and NZL rabbits, analysing the relationship between the differences in production performance and the intestinal microbial community of different breeds. We observed that the production performance of SC rabbits was lower compared to NZL rabbits, but the disease resistance of SC rabbits was better compared to NZL rabbits under the same rearing environment, which is consistent with the breed characteristics of the local breed. Chen et al. (2017) pointed out in their study that increasing the diversity of the intestinal flora of young rabbits during weaning could reduce the rate of diarrhoea and increase the rate of weaning. In this study, the community richness based on alpha diversity did not show any significant differences between the two groups of rabbits, but the SC rabbit group contained a higher number of microbial taxa than the NZL rabbit group. In conclusion, this result suggests that SC rabbits have greater resistance to intestinal disturbance and can adapt to a more complex environment than the NZL rabbit group.

Analysis of the composition of rabbit caecum flora

In the present study, the dominant caecum flora in the two groups of rabbits was *Firmicutes* and *Bacteroidetes*. However, the difference in *Firmicutes* was not significant, which is consistent with the study of Eshar and Weese (2014). *Bacteroidetes* belong to many fibre-degrading bacteria that have stably colonized the host caecum for a long time and are more stable than *Firmicutes* (Faith et al. 2013). *Bacteroidetes* have a significant advantage in carbon utilization and carbohydrate degradation, being able to degrade plant or animal glycosides and produce large amounts of short-chain fatty acids to provide nutrients and calories to the host (Koropatkin et al. 2012). Researches showed that *Bacteroides* was believed to be largely related to the pectin dissolving activity in the caecum of rabbits, and that the decomposed small-molecule sugars could promote the growth and colonization of *Bifidobacteria* so as to promote intestinal health (Rogowski et al. 2015). SC rabbit is a local breed with unique species. In this study, the relative abundance of *Bacteroides* in the caecum in the SC rabbit group was higher than that in the NZL rabbit group, which may be related to the rough feeding tolerance of SC rabbits.

The composition of rabbit intestinal flora is affected by various factors such as breed and feeding environment etc. Healthy animals maintain a dynamic balance of intestinal flora, while dysbiosis of intestinal flora can induce diseases in various aspects. *Proteobacteria* contain various pathogens such as *Escherichia coli, Salmonella*, and *Vibrio cholerae*, and are associated with severe intestinal inflammation, like intestinal disease and necrotizing enterocolitis (Shin et al. 2015). Perinatal exposure to *Proteobacteria* can easily induce epidemic enteropathy and respiratory disease in young rabbits (Turturice et al. 2018). The increasing proportion of *Proteobacteria* in the intestinal flora will lead to an increase in the level of toxins and inflammatory factors in the organism, which in turn causes the disease (Musso et al. 2010; Zhao 2013). Rhee et al. (2004) found that *Bacteroides fragilis* could promote the development of the intestinal immune system, boost the expression of caecal defence genes, effectively inhibit the invasion of pathogenic microorganisms, and have the effect of preventing enteritis. *Bacteroides* is considered to be potentially an important component of beneficial microorganisms. *Bacteroides* is capable of meeting the own energy requirements for growth. When *Bacteroides* is in the absence of other carbon sources, it will break down intestinal mucin and will not directly attack the host intestinal barrier to cause the disease (Zitomersky et al. 2013). In this study, the relative abundance of harmful bacterial groups such as *Vibrio cholerae* and *Proteobacteria* in the SC rabbit group was significantly lower than that in the NZL rabbit group,

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while the content of beneficial bacterial genera such as *Bacteroidetes* and *Ackermania* in the SC rabbit group was higher than that in the NZL rabbit group. These results are consistent with the reduced prevalence of SC rabbits, which may explain the disease resistance of local breeds.

Gene function prediction analysis

The PICRUSt gene prediction shows that the most important functional categories of caecum microflora are energy metabolism, glycolysis pathway, carbohydrate metabolism, fat metabolism, and amino acid metabolism in the two domestic rabbit breeds. The caecum microflora in rabbits can play a role in the digestion, metabolism of nutrients and affect their growth performance. The difference in the carbohydrate metabolism of caecal microorganisms can explain the phenomenon that SC rabbits are better than NZL rabbits in roughage tolerance. However, it needs to be further verified whether these differences are the main reasons.

CONCLUSION

Overall, the caecum flora of the two rabbit species not only differed in species abundance but also shared a variety of common core flora. Compared with NZL rabbits, the intestinal flora structure of SC rabbits was optimized, the relative abundance of harmful flora was significantly reduced, and a healthier intestinal flora structure was established, which enhanced tolerance to roughage. On the study of the intestinal flora of SC rabbits, this paper will help deepen the understanding of intestinal flora of local domestic rabbit breeds, providing necessary data for further utilization of SC rabbits and the development of feeding standards.

Acknowledgement

We thank to College of Animal Science and Technology, Southwest University for valuable help.

Conflict of interest

The authors declare no conflict of interest.

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Received: August 28, 2020
Accepted: May 5, 2021
Published online: June 25, 2021