

# The 16S rRNA gene sequencing of gut microbiota in chickens infected with different virulent Newcastle disease virus

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## Research Article

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# Abstract

## Background

Newcastle disease virus (NDV) is pathogenic to chickens, which is characterized by dyspnea, diarrhea, nervous disorder and hemorrhages. However, the influence of different virulent NDV infection on the host gut microbiota composition is still poorly understood. In this study, twenty 21-day-old specific pathogen free chickens were inoculated with either the velogenic Herts33 NDV strain, lentogenic La Sota NDV strain or sterile phosphate buffer solution (PBS). Through 16S rRNA sequencing, the collected fecal samples of control and NDV infected chickens were examined.

## Results

The results showed that the gut microbiota were mainly dominated by *Firmicutes*, *Bacteroidetes* and *Proteobacteria* in both healthy and NDV infected chickens. NDV infection altered the structure and composition of gut microbiota. As compared to PBS group, phylum *Firmicutes* were remarkably reduced, whereas *Proteobacteria* was significantly increased in velogenic NDV infected group. While the gut community structure has no significant differences between lentogenic NDV infected group and PBS group at phylum level. At genus level, *Escherichia-Shigella* was significantly increased in both velogenic and lentogenic NDV infected groups, but the *Lactobacillus* was only remarkably decreased in velogenic NDV infected group. Collectively, different virulent NDV infection resulted in a different alteration of the gut microbiota in chickens, including a loss of probiotic bacteria and an expansion of pathogenic bacteria.

## Conclusion

These results indicated that NDV with different virulence have different impact on chicken gut microbiota and may provide new insights into the intestinal pathogenesis of NDV.

## Background

All vertebrate animals are inhabited by an immense population of microorganisms. The intestinal tracts maintain a particular rich and diverse microbial community with the number over trillions and species more than 1000 [1, 2]. These amazing amount of gut microbes were previously thought to be mainly benefit for food sources utilization. Recently, with the developing of research, scholars found that these microbes also play an essential role in many aspects of the host's physiology, including nutrients digestion, immune system development, detoxification of some compounds, and resistance to pathogens [3–6]. Although the diversity, the roles and the importance of these microbes in animal's physiology have been illustrated, the biological significance of the presence of intestinal microbes in animals remains largely unclear. As the unique life history trait of birds that are different from other vertebrates, such as hatching from eggs, chickens are an interesting study object for intestinal microbes. However, research of the avian intestinal microbiota was thought to be relatively fallen behind that of other vertebrates and the recent study about avian intestinal microbiota mainly focused on composition of gut microbiota at different development stages, different

segments of gut, and different living condition. Little is known about the interaction between viral infection and avian gut microbiota. Existed reports were limited to avian influenza virus, infectious bronchitis, marek's disease virus, infectious bursal disease virus and Newcastle disease virus [7–14]. With the ongoing prohibition of using antibiotic as growth promoter and the recognition of benefits of a healthy gut microbiota played in promotion growth and resistance of viral and bacterial diseases [15, 16], extensive study is still required to understand more about the interaction between virus infection and gut microbiota.

As a highly contagious avian disease, Newcastle disease (ND) causes hemorrhages and necrosis of the respiratory tract and the digestive tract, result in high morbidity and mortality in chicken and has caused great economic losses to the poultry industry. Newcastle disease virus (NDV), the causative agent of ND, belongs to the family *Paramyxoviridae* and has a single-stranded, non-segmented, negative-sense RNA genome. It's genome is approximately 15.2 kb in length and contains six genes in the order of 3'-NP-P-M-F-HN-L-5' [17]. According to the disease severity in chicken post infection, NDV strains are categorised as three pathotypes: highly pathogenic (velogenic) strains, which exhibit systemic infections with high mortality, including intestinal symptoms; intermediate (mesogenic) strains, which show intermediate virulence; and apathogenic (lentogenic) strains, which cause mild or asymptomatic infections that are restricted to the respiratory tract [18]. La Sota is a naturally occurring lentogenic NDV strain. Because of the good safety and efficacy, it has been widely used as a live vaccine to prevent Newcastle disease outbreaks in poultry practice. While, Herts33 is a velogenic strain isolated from fowl in 1933. Previously, Cui *et al.* reported that lentogenic NDV infection interferes with the formation of intestinal microbiota in newly hatched chicks by 16S rRNA gene sequencing technology [13]. But does the impact of different virulent NDVs on chicken gut microbiota are same is still unknown and need further investigation. Here, we evaluated the influence of different virulent NDV on gut microbiota composition in 21-day-old specific pathogen free chickens by 16S rRNA sequencing technology. To our knowledge, this is the first report that illustrate the impact of different virulent NDV on chicken gut microbiota.

## Materials And Methods

### Viruses

NDV strains La Sota and Herts33 were used in the present study. The La Sota strain is a class II genotype II lentogenic strain, and the Herts33 is a class II genotype IV virulent strain. These two strains were propagated in the allantoic cavity of 9-11 day-old embryonated specific pathogen-free (SPF) chicken eggs. Allantoic fluid was harvested from chicken embryos and stored at -70°C. The virus median tissue culture infective dose (TCID<sub>50</sub>) was tested on DF-1 cell by Reed-Muench method.

### Ethical statement

The experiments were performed in strict accordance with Animal Ethics Procedures and Guidelines of the Ministry of Health in China and the ARRIVE guidelines. All experimental procedures were approved and supervised by the Ethics Committee for the Care and Use of Laboratory Animals in Qinghai University, China. Informed consent was obtained from the JINAN SAIS POULTRY CO., LTD in advance.

## Experiment design

Twenty 2-week-old specific pathogen free white Leghorns chickens were purchased from JINAN SAIS POULTRY CO., LTD. The chickens were maintained in bio-security isolation units with feed and water administered *ad libitum*. After acclimatizing for 1 week, all chickens were divided into three groups with seven birds in two experiment groups and six birds in control group, namely group Herts33 (n=7), group La Sota (n=7) and group PBS (n=6). Each bird in groups Herts33, and La Sota was challenged with  $10^5$  TCID<sub>50</sub>/100  $\mu$ L of the Herts33 strain or La Sota strain via eye drop (50  $\mu$ L) and intranasal (50  $\mu$ L) routes (EI/IN), respectively. Birds in the PBS group were challenged with 100  $\mu$ L of PBS. All birds were monitored daily for clinical signs (depression, respiratory signs, diarrhea, etc), mortality. Cloacal swabs were used to collect about 200 mg fecal sample from each bird at 3 to 5 days post challenge for fecal DNA isolation.

## DNA extraction and library construction

Total genomic DNA was extracted from about 200 mg collected feces using QIAamp 96 PowerFecal QIAcube HT kit (QIAGEN) following the manufacturer's instructions. The concentration and purity of extracted DNA was verified with NanoDrop and agarose gel. Then the genome DNA was used as template to amplify V3-V4 variable regions of 16S rRNA genes with universal primers 343F 5'-(TACGGRAGGCAGCAG)-3' and 798R 5'-(AGGGTATCTAATCCT)-3' and Tks Gflex DNA Polymerase (Takara). PCR were carried out in a 30  $\mu$ L reaction mixture containing 2 $\times$  Gflex PCR buffer 15  $\mu$ L, primer 343F (5 pmol/ $\mu$ L) 1  $\mu$ L, primer 798R (5 pmol/ $\mu$ L) 1  $\mu$ L, Tks Gflex DNA Polymerase 0.6  $\mu$ L, and 50 ng DNA template. The PCR condition were initial denaturation at 94°C for 5 min, followed by 26 cycles of denaturation at 94°C for 30 s, annealing at 56°C for 30 s and extension at 72°C for 20 s, with a final extension phase at 72°C for 5 min. The PCR products were visualized using gel electrophoresis, and purified with AMPure XP beads (Agencourt). The purified first round PCR product was used as template to conduct second round PCR with the index primer pairs adapter I5 primer and adapter I7 primer. The PCR reaction system was carried out in a 30  $\mu$ L reaction mixture as the first round PCR. The PCR condition were same as the first round PCR except for the cycles reduced to seven. After purification with the AMPure XP beads again, the final amplicon was quantified using Qubit dsDNA assay kit. Equal amounts of purified amplicon were pooled for subsequent sequencing using Illumina MiSeq system by oebiotech (Shanghai, China).

## Bioinformatic analysis

Raw sequencing data were in FASTQ format. Paired-end reads were then preprocessed using Trimmomatic software to detect and cut off ambiguous bases (N). We also cut off low quality sequences with average quality score below 20 using sliding window trimming approach. After trimming, paired-end reads were assembled using FLASH software. Assemble parameters were: 10 bp of minimal overlapping, 200 bp of maximum overlapping and 20% of maximum mismatch rate. To obtain high quality clean tags, quality filtering of the raw tags was performed using QIIME software (version 1.8.0) quality-controlled process. The chimeric sequences were removed by using the UCHIME Algorithm. Then the clean reads were clustered to generate operational taxonomic units (OTUs) using VSEARCH software with 97% similarity. The representative read of each OTU was selected using QIIME package. All representative reads were annotated and blasted against Silva database Version 123 using RDP classifier (confidence threshold was 70%). In the

present study, all sequences have been deposited to the National Center for Biotechnology Information (NCBI) database under accession number PRJNA700718.

## Statistical Analyses

Differences between populations had been analyzed using parametric (ANOVA) and non-parametric statistical methods. All results were presented as the mean value ( $\pm$  SE). Differences between groups were declared significant at  $P < 0.05$ .

# Results

## Sequencing results overview

In the present study, twenty fecal samples (seven Herts33 challenged, seven La Sota challenged, six PBS negative control) were collected and processed for 16S rRNA gene sequencing and analysis. After quality control, about 60617 to 72639 clean tags were obtained. And the valid tags were distributed between 46932 and 69273 post removing chimera. The average length of valid tags is 406.47 to 425.62 bp and the OUT number of each sample was distributed between 471 and 1477. The Good's coverage ranged from 99.32% to 99.59%, indicating a good sequencing depth enough to cover the majority of the gut microbiota in each sample.

## A decrease in the microbial diversity in gut microbiota with NDV infection.

The fecal sample richness was evaluated by operational taxonomic units (OUT) counts in each sample. As shown in figure 1, the average number of observed OUT in the NDV infected chicken samples was more than that of the PBS control group (Fig.1 Venn plot. Herts 2227, La Sota 1861, PBS 1802).

To evaluate the influence of NDV infection on gut microbiota diversity and richness, Chao index, Shannon's and Simpson's indices were calculated and these indices of each sample were shown in table 1. As shown in figure 2a, the average Chao index of herts33, La Sota and PBS group was 834.884, 878.283 and 725.671, respectively. And a slightly higher Chao index suggested that NDV infection increased the community richness compared to that in the PBS group. However, Wilcox test showed that the chao index had no significant differences from each other (Wilcox,  $P > 0.05$ ). In addition, neither the Shannon nor the Simpson indices were significantly different among these three groups (Wilcox,  $P > 0.05$ ). The higher Shannon and Simpson index indicated a higher bacterial diversity, which meant that the virulent Herts33 NDV infection decreased the microbial diversity compared to that in the avirulent La Sota group and PBS control group (Fig.2b and Fig.2c).

## Gut bacterial beta-diversity analysis

To analysis the similarities and differences of bacterial communities among these three groups chickens, the Bray-Curtis similarity were calculated. And the Bray-Curtis based analysis of similarities indicated that the microbiota among three groups were significantly different from each other ( $R = 0.3908$ ,  $P = 0.001$ ). Furthermore, principal coordinate analysis (PCoA) were performed based on Bray-Curtis distances to visualize

the similarity of the microbial community structure in different groups. As shown in figure 3, PC1 and PC2 account for 33.23 and 18.89% of the total variation. And there was distinguishing clustering of the samples from each group. However, partial samples from La Sota and PBS were close to each other. The PCoA result suggested distinct differences in the bacterial composition among the three groups.

### **NDV infection alter the gut microbiome composition in chickens**

To elucidate the effect of NDV infection on gut bacterial composition, we evaluated the gut microbiota at different taxonomical levels. The overall bacterial composition of different groups at the phylum level was showed in figure 4A and 4a, sequences that accounted for very small proportions were combined as others. From figure 4A and 4a, we found that *Firmicutes*, *Proteobacteria* and *Bacteroidetes* were the three most abundant phyla in all groups. The average relative abundance of phylum *Firmicutes* in Hetts33 group was significantly lower than that in other two groups (Fig.5a, ANOVA  $P \leq 0.01$ ), while the relative abundance of phylum *Proteobacteria* was significantly higher than that in La Sota and PBS groups (Fig.5a, ANOVA  $P \leq 0.01$ ).

When analyzed at the genus level, as shown in figure 4B and 4b, the main genera in these three group included *Lactobacillus*, *Escherichia-Shigella*, *Enterococcus* and *Bacteroides*. The top 10 significantly different genus were *Lactobacillus*, *Escherichia-Shigella*, *enterococcus*, *GCA-900066575*, *Clostridium*, *Pseudomonas*, *Azospirillum*, *Pseudogracilibacillus*, *Weissella* and *Brachybacterium* (Fig.5b, ANOVA,  $P \leq 0.05$ ). The relative abundance of genus *Lactobacillus* in Hetts33 group was significantly lower than other two groups. While the relative abundance of genus *Escherichia-Shigella* in Herts33 group and La Sota group was significantly higher that of PBS group (Fig.6a and 6b, T test  $P < 0.05$ ). However, the abundance of genus *Lactobacillus* had no significant difference between La Sota group and PBS group (Fig.6b, T test  $P > 0.05$ ). And the relative abundance of genus *enterococcus* in hetts33 group and La Sota group was significantly lower that of PBS group.

## **Discussion**

The intestine tract of chickens is populated with a relatively rich and diverse microbial community, including bacteria, viruses, fungi and protozoa, as other animals. These incredibly complex microbial community possess important functions for host on many aspects. At the same time, the intestinal microbiota is dynamic and influenced by environment, diet, age, antibiotics, pathogen infection and other factors [19]. So the maintenance of a health gut microbiota is very important and contributes significantly to the overall health and performance of a flock [20]. If the structure and composition of gut microbiota is disturbed, this may have a severe impact on the chickens' grow performance and may enhance the risk for systemic diseases including infectious diseases [21]. Viruses and bacteria could interact with each other in the gut, and thus affect the virus replication and transmission [22,23]. Therefore, this study was designed to evaluate whether NDV infection could cause the alteration of chicken gut microbiota.

Previous study reported that the infection of NDV resulted in the disproportion of intestinal microbiota [13]. In the present study, we compared the gut microbiota between different virulent NDV infected chickens and non-infected chickens by 16S rRNA gene sequencing and found that NDV could alter the gut microbiota composition at different levels, which is in line with previous observations [13]. To examine whether vertical

infection of NDV influence the formation of intestinal community, Cui evaluated the effect of NDV infection on chick embryos at hatch. Their result showed that NDV infection decreased the richness and overall diversity of duodenal flora, but the richness and diversity of cecal microflora was not affected. Our result is in accordance with Cui's result on cecal as the alpha diversity indexes were not significantly different between NDV infection groups and control group in the present work. The results of PCoA indicated that the NDV infection altered the structure of gut microbiota, which is consistent with the results of previous study [13]. And from the PCoA results we concluded that different virulent NDV infection have a vary influence on chicken microbiota.

Many studies have demonstrated that chicken gut microbiota consisting of three major bacterial phyla, namely the *Firmicutes*, the *Proteobacteria*, and the *Bacteroidetes*. Our present study also found that the above three bacterial phyla were the predominant observed bacterial taxa, which confirmed previous observations [24]. However, the relative abundances of these three phyla was quantitatively different among our three groups. In PBS control group, the *Firmicutes*, the *Bacteroidetes*, and the *Proteobacteria* accounted for 80.98%, 16.07% and 2.31% of the total bacterial, respectively. While the proportion of *Firmicutes*, *Bacteroidetes*, and *Proteobacteria* in the velogenic Herts33 NDV infected group was 13.41%, 10.73% and 68.35%, and in the lentogenic La Sota NDV infected group was 74.12%, 3.7% and 21.37%. The functions of *Firmicutes* and *Bacteroidetes* are closely related with carbohydrate and protein metabolism and paly a role in energy production [25,26]. At the same time, some members in phyla *Firmicutes* could regulate the inflammation by induction of anti-inflammatory cytokines [27]. As a minor constituent in the fecal microbial community, the *Proteobacteria* accounted for only 2.31% in PBS group (Fig. 5) and this group included many pathogenic bacteria, such as *Escherichia*, *Shigella*, *Salmonella*, *Clostridium Cluster XI*, *Vampirovibrio* and so on [28]. As compared to PBS group, the increase of the *Proteobacteria* and decrease of *Firmicutes* in two NDV infected group (velogenic Herts33 VS PBS,  $P < 0.01$ ; lentogenic La Sota VS PBS,  $P > 0.05$ ) may be a sign of disease in chickens.

*Lactobacillus* are one of the predominant bacterial genera in the gastrointestinal tract of chicken [24], and great benefits for chickens, such as help in carbohydrate fermentation, restriction of the replicate of other bacteria species by production of lactate, bacteriostatic and bactericidal substances [29-32]. In addition, *Lactobacillus* could modulate the immune system and significant enhancement of the immune response was also observed in chicken [33]. Now, *Lactobacillus* strains are actually considered as safe organisms and has been widely used as a probiotics to improve grow performance and inhibit the potential pathogenic microorganisms such as *Salmonella* and *Escherichia-coli* [34,35]. In this study, as the most abundant genus and top one different genus, the relative abundance of *Lactobacillus* in velogenic NDV infection group was significantly lower than that of PBS group (T test,  $P < 0.01$ ), but that has no significant differences between the lentogenic NDV infection group and PBS group (T test,  $P > 0.05$ ). The decline of *Lactobacillus* were also observed in chickens post *Eimeria tenella* or H9N2 avian influenza virus infection [8,36]. It has been shown that some *Lactobacillus* can enhance the IFN and IL-22 production and response [37,38], and higher abundance of the *Lactobacillus* was associated with restoration of the epithelial barrier integrity [39,40]. Furthermore, Oral administration of *Lactobacillus* can effectively relieve diarrhea by regulating intestinal microflora and improving immune system function [41]. So we speculated that the differences in the abundance of *Lactobacillus* post different virulent NDV infection would account for different clinical signs

and pathogenicity in intestinal. The detail mechanisms of why different virulent NDV have different influence on the quantity of *Lactobacillus* and what pathway does NDV use to affect the *Lactobacillus* need further investigation.

In contrast, opportunistic pathogen *Escherichia-Shigella*, which belongs to family *Enterobacteriaceae*, was significantly increased both in two NDV infected groups. In velogenic NDV infected group, the average relative abundance of *Escherichia-Shigella* increase from 1.4% to 53.3%, while that increased from 1.4% to 19.8% in lentogenic NDV infected group. The increasement of *Escherichia-Shigella* was also observed in the infection of H9N2 avian influenza virus, ALV-J, duck reovirus and *Eimeria tenella* [42,43]. And some reports suggested a positive correlation between the abundance of *Escherichia-Shigella* and the development of necrotic enteritis in chickens [44]. Moreover, previous studies have shown found that IFN- $\alpha$ , IFN- $\beta$ , IFN- $\gamma$ , and IL-22 expression were negatively correlated with *Clostridium cluster-XI*, *Escherichia*, and *Shigella* species post AIV infection [9,37]. In H9N2 AIV infected chickens, elevated level of IFNs caused the dysbiosis of commensal gut microbiota and decreased the number of lactic acid producing bacteria due to an increased relative abundance of pathogenic *Proteobacteria*, including *Shigella*, which produce inflammation in GIT [45]. These date indicate that NDV infection might increase the possibility of subsequent infection by other pathogens. Does the different expression level of cytokines, such as IFNs, IL22, IL17, which were induced by NDV infection, account for differences in gut microbiota alteration and clinical symptoms post different virulent NDV infection need further study.

## Conclusions

In conclusion, our study demonstrated that significant dysbiosis occurs in the gut microbiota of chickens post NDV infection. The alteration of gut microbiota was dominant by an increased relative abundance of the pathogen *Escherichia-Shigella*, and apparent decrease in the level of the non-pathogenic bacteria, for example *lactobacillus*. These observation indicate that a fundamental alteration in the chicken gut microbiota post NDV infection. Further investigation of the mechanisms underlying these interactions could help reveal useful targets ant treatment approaches for restoring the gut microbiota to help combat NDV.

## Declarations

### Acknowledgements

Not applicable.

### Authors' contributions

LNT designed the study, carried out the experiments, analyzed data and wrote the manuscript. SHR, JLW and JW helped perform the experiments. YQ helped in provide reagents and materials. WW, Adam FEA and ZKL helped revised the manuscript. ZQY and XLG designed the study and draft the manuscript. All authors read and approved the final manuscript.

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### **Availability of data and materials**

All data generated or analyzed during this study are included in this published article, and also available from the corresponding author on reasonable request.

### **Ethics approval and consent to participate**

The experiments were performed in strict accordance with Animal Ethics Procedures and Guidelines of the Ministry of Health in China and the ARRIVE guidelines. All experimental procedures were approved and supervised by the Ethics Committee for the Care and Use of Laboratory Animals in Qinghai University, China. Informed consent was obtained from the animal owners in advance.

### **Consent for publication**

Not applicable.

### **Competing interests**

The authors declare that they have no conflict of interests.

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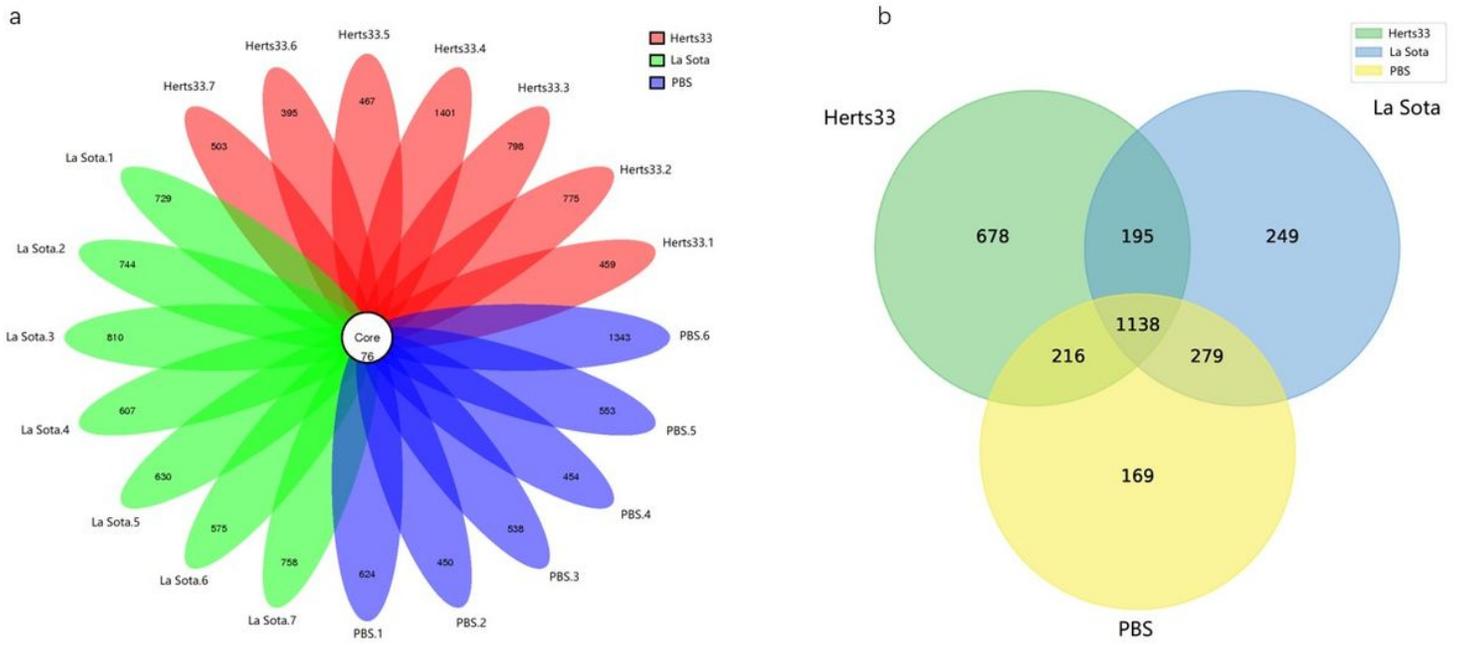
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# Table

Table 1  
The estimators of sequence diversity and richness

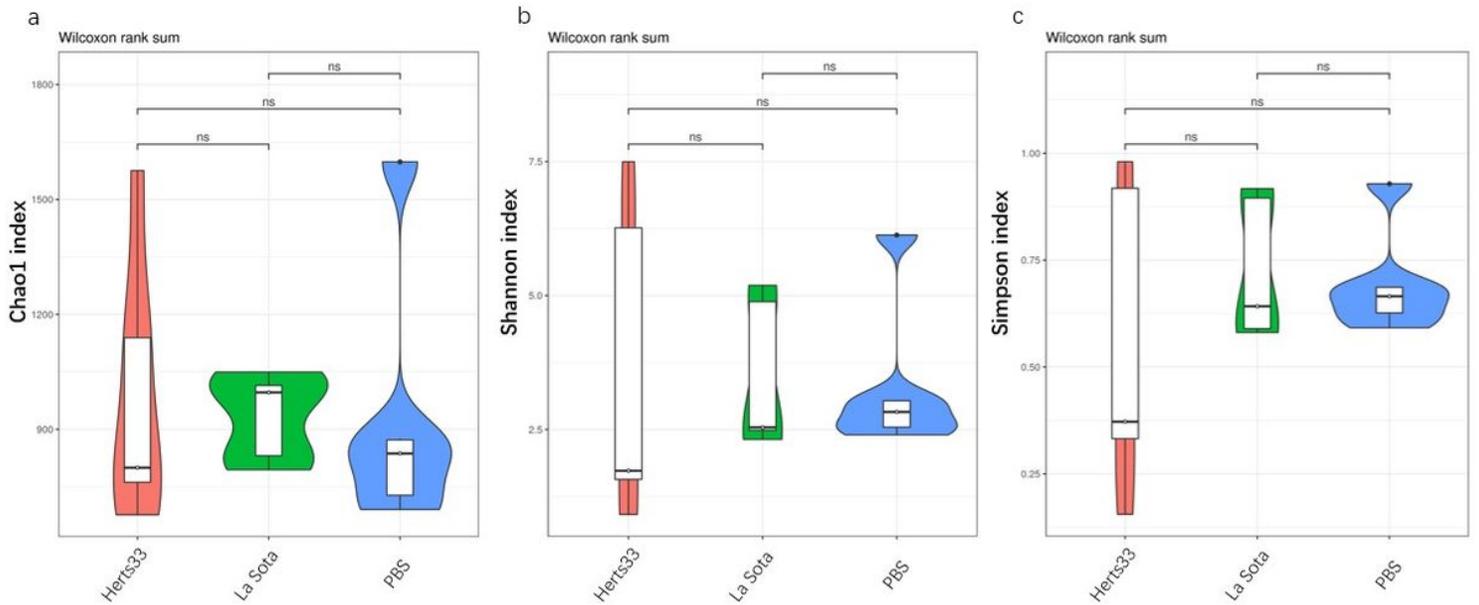
Samples	Reads	OTUs	simpson	chao1	Observed species	shannon	Goods coverage	PD whole tree
Hetts33.1	67293	535	0.315108	764.6402	447.8	1.40745	0.995131	21.40172
Hetts33.2	54131	851	0.917592	1126.855	815.2	5.980166	0.995479	36.46119
Hetts33.3	57483	874	0.918702	1151.576	820.5	6.545797	0.995068	37.76043
Hetts33.4	50601	1477	0.980155	1575.387	1458.1	7.497044	0.995838	57.57711
Hetts33.5	69273	543	0.155358	759.3362	449.8	0.914995	0.995384	21.97474
Hetts33.6	61314	471	0.372028	677.053	416.3	1.730422	0.995881	20.37164
Hetts33.7	63697	579	0.349542	799.8134	501.5	1.730623	0.99504	24.97967
La Sota.1	58778	805	0.913189	1006.878	746.1	5.120451	0.99454	30.27542
La Sota.2	58034	820	0.9171	1049.569	756.2	5.189811	0.994462	30.44099
La Sota.3	61316	886	0.584854	1022.178	815.2	2.510756	0.994423	35.28361
La Sota.4	64931	683	0.594488	824.6888	609.2	2.538315	0.995507	27.8734
La Sota.5	62199	706	0.580574	837.9335	634.3	2.316419	0.99486	27.89631
La Sota.6	60679	651	0.641917	794.4215	593.1	2.462201	0.99524	27.51244
La Sota.7	57813	834	0.877119	996.3602	774.2	4.652152	0.994438	31.2902
PBS.1	61439	700	0.619337	861.2555	630.9	3.045674	0.995023	26.75791
PBS.2	59118	526	0.647619	690.9104	476.7	2.510859	0.995636	21.37007
PBS.3	60339	614	0.683144	876.2117	544.2	2.643193	0.994499	24.31326
PBS.4	62201	530	0.687748	699.7192	463.5	2.40015	0.995292	22.87679
PBS.5	55910	629	0.591706	812.8232	585.7	3.014565	0.995425	24.2154
PBS.6	46932	1419	0.928459	1598.205	1411.9	6.129219	0.993234	51.03208

# Figures



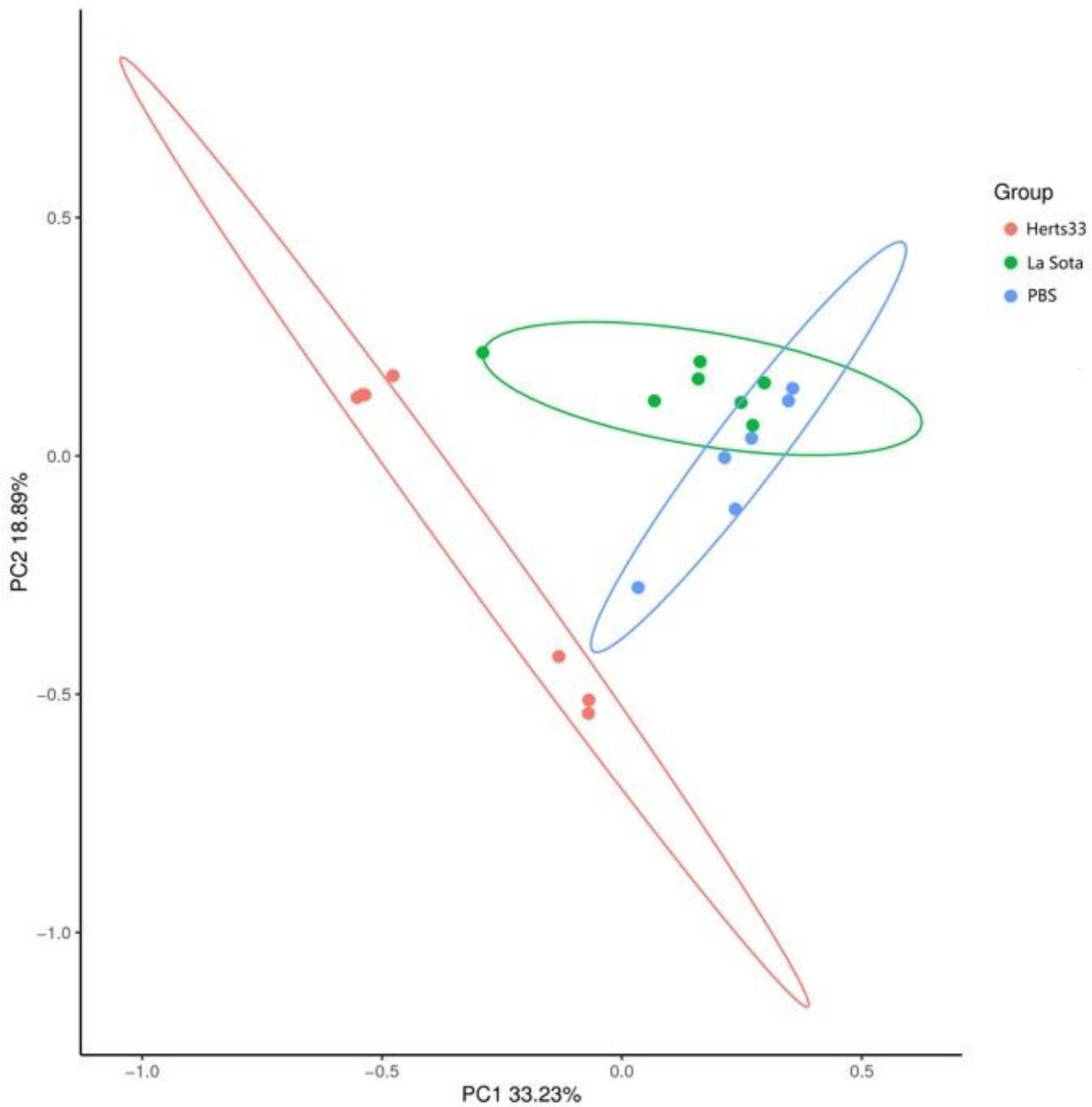
**Figure 1**

The community composition analysis. a Venn diagram showing overlap in OTUs of differential abundance of each samples in Herts33, La Sota and PBS groups. b Venn diagram showing overlap in OTUs of differential abundance of in Herts33, La Sota and PBS groups.



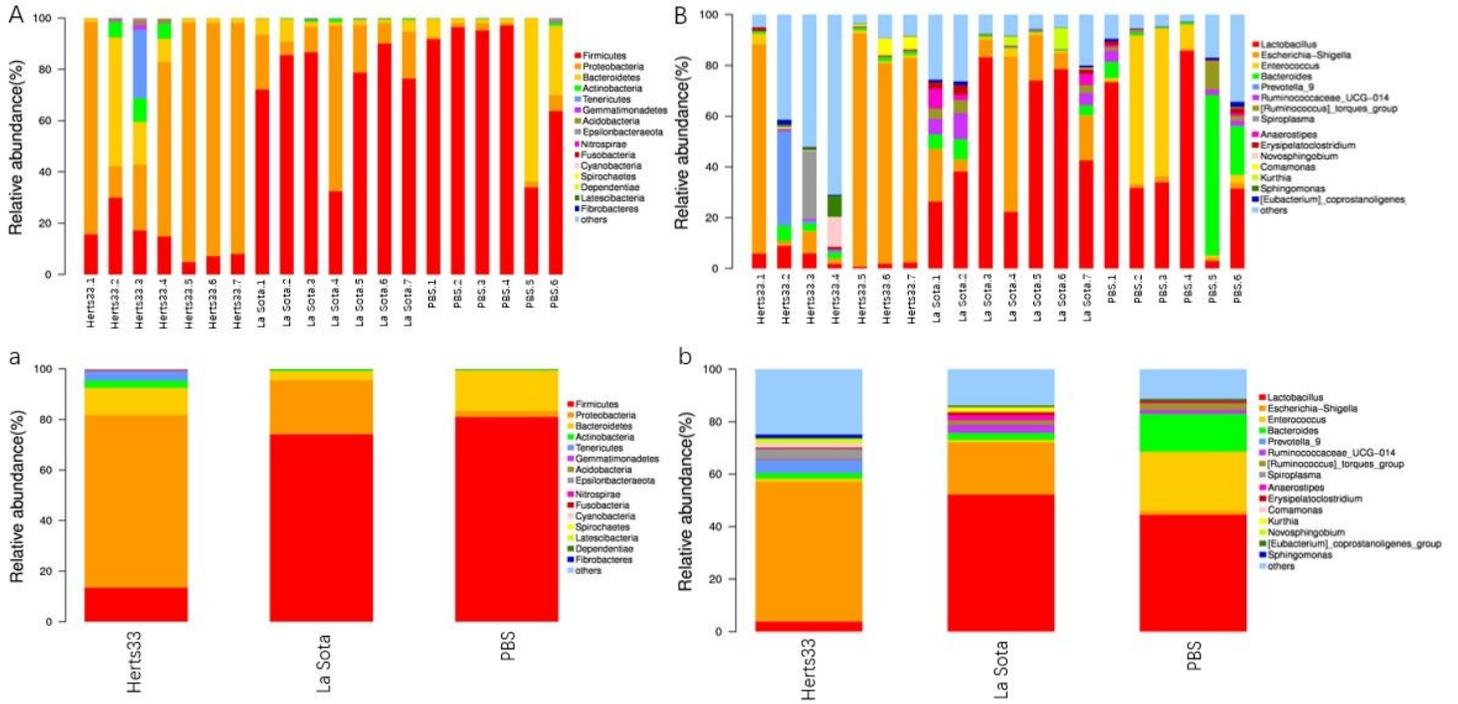
**Figure 2**

The microbial diversity index analysis. a Chao1 index. b Shannon index. c Simpson index.



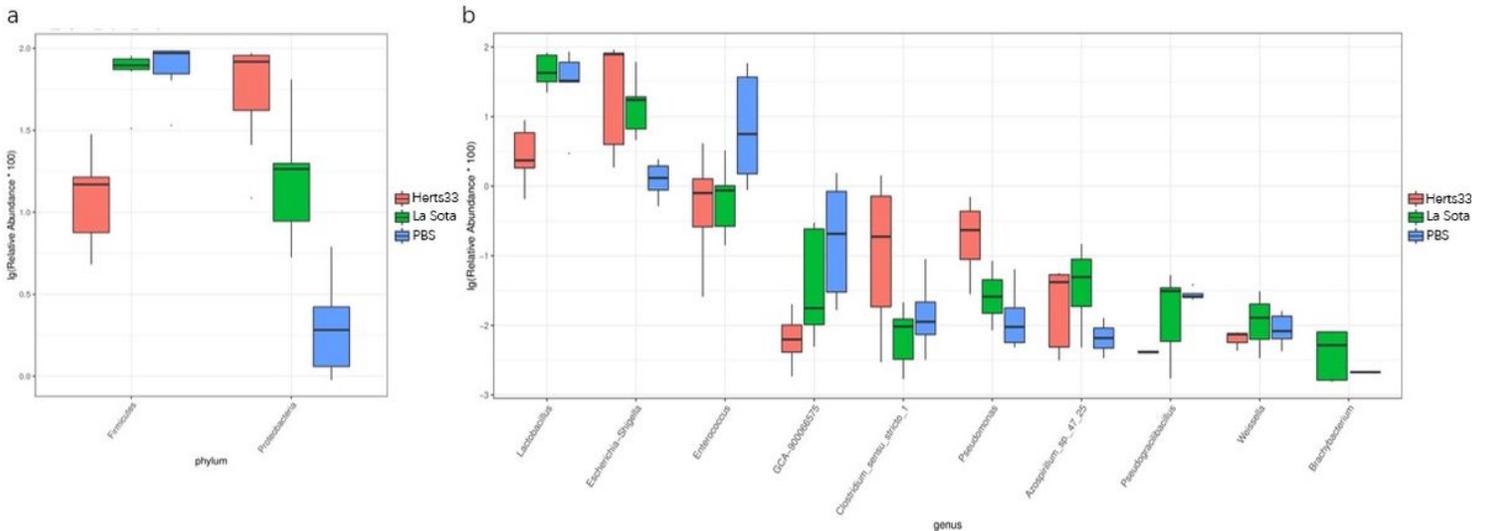
**Figure 3**

PCoA analysis of similarities between different groups. Principal component (PC) 1 and 2 accounted for 33.23% and 18.89% of the variance, respectively.



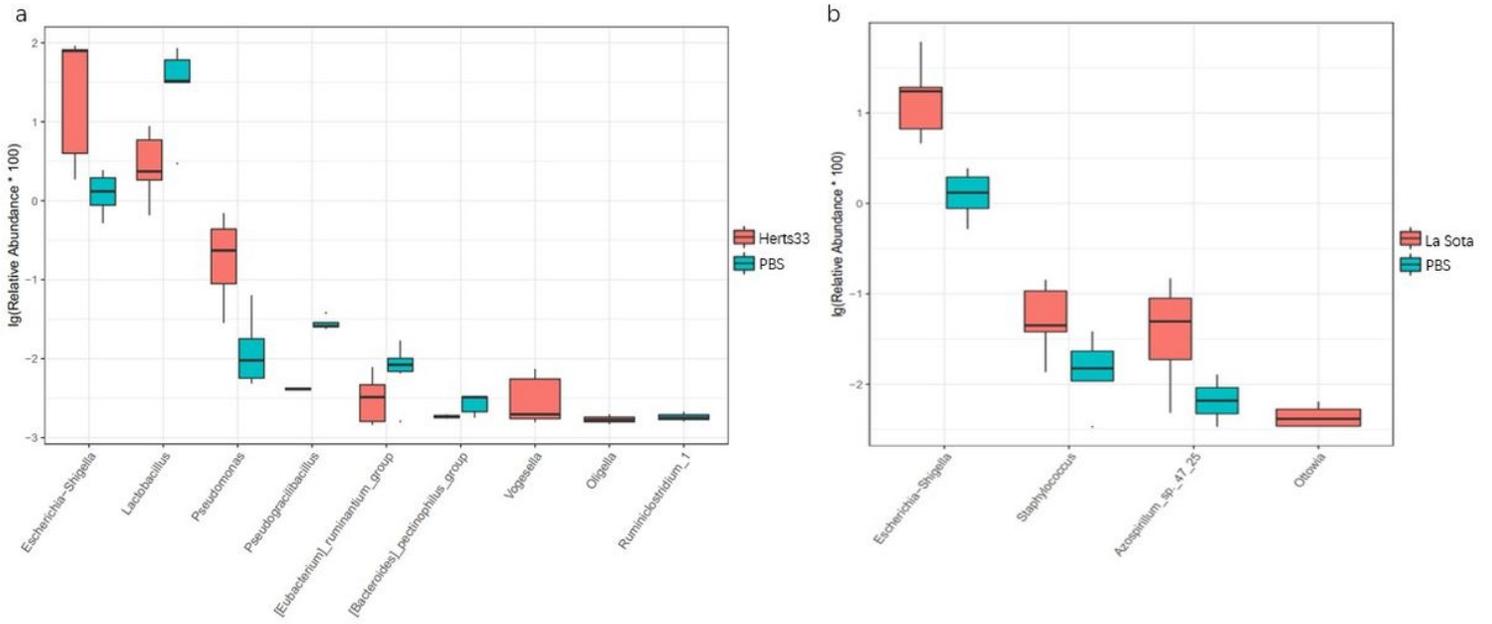
**Figure 4**

Microbial composition of different samples and groups. Each bar represents the relative abundance of each bacterial taxon within samples or groups. A Taxa assignments of each sample at Phylum level. a Taxa assignments of each group at Phylum level. B Taxa assignments of each sample at Genus level. b Taxa assignments of each group at Genus level.



**Figure 5**

The relative abundance of top 10 different bacteria in three groups (ANOVA), expressed as an average percentage of the total. a At phylum level. b At genus level.



**Figure 6**

The relative abundance of top 10 different genera bacteria between NDV challenge groups and control group(T test), expressed as an average percentage of the total. a Herts33 VS PBS. b La Sota VS PBS.