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L-Asparagine is the essential factor for the susceptibility of Chinese pigs to *Mycoplasma hyopneumoniae*

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Abstract

Swine mycoplasma pneumonia caused by *Mycoplasma hyopneumoniae* is the most prevalent and frequently occurring chronic respiratory disease in pigs worldwide. Pig breeds in China are more susceptible to *Mycoplasma hyopneumoniae*, and understanding the reasons for their susceptibility is crucial for the prevention and control of swine *Mycoplasma* pneumonia, as well as for promoting the healthy development of the swine industry. *Mycoplasma hyopneumoniae* has limited biosynthesis and metabolism, and has harsh requirements for nutrients, thus it is necessary to add approximately 15%-20% porcine serum in medium during in vitro culture, and porcine serum plays an irreplaceable role in the growth and reproduction of *Mycoplasma hyopneumoniae*. In this study, growth titres of different *Mycoplasma hyopneumoniae* strains cultured in the serum medium from different Chinese pig breeds and introduced pig breeds were compared. Non-targeted metabolomics analysis of representative introduced and Chinese pig breeds, Large white pig and Bama miniature pig, respectively, screened of key serum metabolites. The results showed that, expression level of L-Asparagine in Bama miniature pigs was 15–18 times higher than in Large white pigs. Additionally, the contents of inositol and Arachidonic acid in Bama miniature pigs were significantly upregulated as well. A certain concentration of L-Asparagine significantly increased the growth titer, metabolic capacity and pathogenicity of *Mycoplasma hyopneumoniae*. In conclusion, Bama miniature pig and Large white pig are representative pig breeds that are susceptible and non-susceptible to *Mycoplasma hyopneumoniae* respectively. L-Asparagine is the essential factor and biomarker for the susceptibility of Chinese pigs to *Mycoplasma hyopneumoniae*.

Keywords *Mycoplasma hyopneumoniae*, Different pig breeds, Susceptibility, L-Asparagine, Untargeted metabolomics, Biomarker

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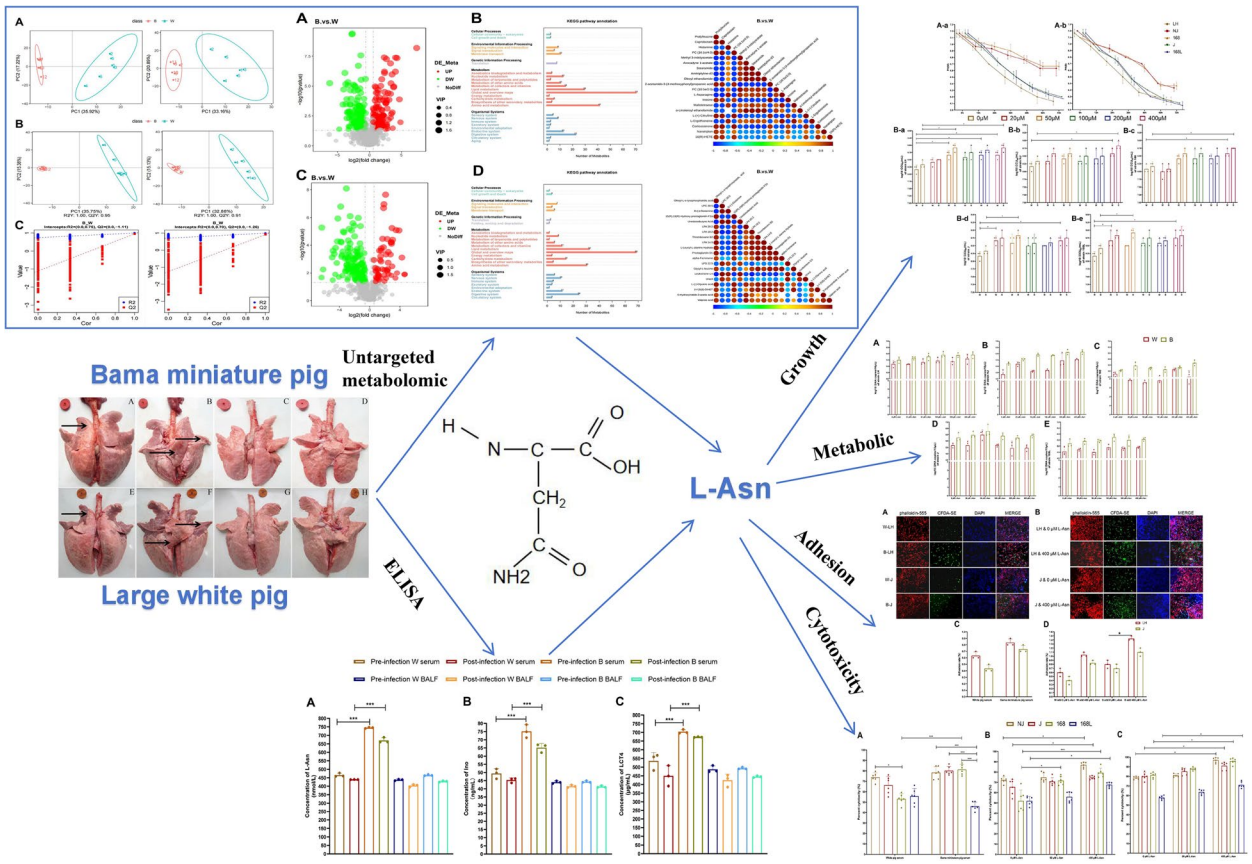
Graphical Abstract



Introduction

Porcine enzootic pneumonia or swine respiratory disease, is a chronic respiratory infectious disease caused by *Mycoplasma hyopneumoniae* (*M. hyopneumoniae*) in pigs. It is characterized by high morbidity, widespread prevalence, difficulty in eradicating, long-term and debilitating effects, and susceptibility to secondary diseases leading to significant economic losses to the pig industry (Maes et al., 2018; Zimmer et al., 2020). Importantly, significant differences existed in the susceptibility to *M. hyopneumoniae* among different pig breeds. For example, Chinese pig breeds such as Bama miniature pigs and Taihu pigs are highly susceptible to *M. hyopneumoniae* infection, with high morbidity and severity, while introduced breeds like Large white pigs, Landrace and Duroc pigs demonstrate relatively lower susceptibility to *M. hyopneumoniae* (Fang et al., 2015; Huang et al., 2017; Ni et al., 2019). By comparing the morbidity and gross lung lesions before and after infection with *M. hyopneumoniae* in local and introduced pig breeds, it was found that the degree of lesions in local pig breeds, Bama miniature pigs, was significantly more serious than that in introduced pig breeds, Large white pigs (Gan et al., 2020).

Mycoplasma including *M. hyopneumoniae*, has a small genome with limited biosynthetic and metabolic capabilities, and has higher nutritional requirements compared to typical bacteria. Gram-positive bacteria such as *staphylococci* grow well in ordinary gravy media, Gram-negative bacteria such as *pneumophila* have low nutritional requirements and form large grayish-white mucus colonies on normal agar medium. However, *Mycoplasma* in vitro culture needs to be supplemented with 10%-20% human or animal serum. Serum contains the necessary cholesterol and saturated/unsaturated fatty acids that are essential for the growth of most *Mycoplasma* during their life cycle (Waites et al., 2017). Amino acids are also indispensable nutritional requirements for *Mycoplasma*, and the specific quantity needed varies among species. The damage suffered by the host is often a byproduct of the pathogen satisfying its own nutritional requirements. For

example, *M. hyopneumoniae* can utilize glycerol to generate hydrogen peroxide, inducing cell toxicity at infection site (Ferrarini et al., 2018).

Metabolomics is an emerging tool that utilizes metabolomics techniques to analyze metabolomes, enabling the screening of potential biomarkers and key metabolic pathways. For example, untargeted metabolomic analysis of serum from piglets infected with *classical swine fever virus* (CSFV) revealed changes in metabolites associated with several key metabolic pathways (Gong et al., 2017). By analyzing metabolites and biomarkers related to key metabolic pathways, biological differences between different pig breeds could be understood (Bovo et al., 2016). Recent research has also indicated the association between metabolic disorders and the pathophysiology of silver viper snakebites, as well as exploring potential diagnostic biomarkers using untargeted metabolomics (Huang et al., 2021). The above studies suggest that searching for corresponding metabolic markers through metabolomics is conducive to research on the susceptibility of corresponding pathogens and species.

In this study, Bama miniature pigs, the representative local breed, with best in vitro culture effect and the most severe morbidity and gross lung lesions with its serum culture medium induced by *M. hyopneumoniae*, growth and pathogenicity were significantly higher than that of serum from introduced breed of Large white pigs. Through untargeted metabolomic analysis of serum from representative local and introduced pig breeds, the effects on the growth, metabolism and pathogenicity of *M. hyopneumoniae* infection were investigated, serum-specific metabolic markers were uncovered, which will partially elucidate the reasons for the greater susceptibility of Chinese pig breeds to *M. hyopneumoniae* compared with introduced pig breeds.

Materials and methods

M. hyopneumoniae strains and growth conditions

Five strains (namely 168, 168L, NJ, J and LH) were cultured and passaged at a ratio of 1:10 in modified Friis medium, designated KM2 cell-free medium, which was supplemented with 20% (v/v) Large white pig serums, and cultivated in a humidified incubator at 37℃(Xiong et al., 2014). *M.hyopneumoniae strain* 168 was isolated and cultured from a pig with typical symptoms of *Mycoplasma pneumonia of swine* (MPS) in China. This field strain was gradually attenuated through continuous passage until the 380th generation, generating the low-virulence strain 168L (Liu et al., 2013), and the *M. hyopneumoniae* 168L strain used in this study was 353rd generation. The pathogenic strain NJ was isolated in Nanjing city, and was confirmed it can induce histopathological lesions when it was nasally challenged in mice (Zhang et al., 2021). Strain J (ATCC 25934) was passaged for three generations to obtain frozen stocks. Strain LH was also a virulent clinical strain and capable of inducing typical characteristics of EP (GenBank accession number: CP079799) (Xie et al., 2021).

Serum collection and storage

Every six blood and bronchoalveolar lavage fluid (BALF) samples were collected from 2.5-month-old male local pig breeds of Bama miniature pigs (average weight of 8.5 kg) and introduced pig breeds of Large white pigs (average weight of 20 kg) with the same feeding conditions 28 days before and after *M. hyopneumoniae* infection. Serum were separated from whole blood by centrifugation at 1000 × g and 4 °C for 10 min and stored in 1 mL aliquots at − 70 °C for later use.

Before detailed analysis, antibody test based serological analysis showed that all the following pathogens were negative, including *M. hyopneumoniae*, *pseudorabies virus*, *classical swine fever virus*, *type O foot and mouth disease virus*, *porcine respiratory and reproductive syndrome virus* (PRRSV), *swine influenza virus* of H1 and H3 subtype and *porcine circovirus type 2* (PCV2) (Xie et al., 2022). Under the same conditions, every six blood samples from local breed Meishan pigs imported breed Duroc pigs were also collected as above mentioned, collection method of BALF was according to our previous study (Gan et al., 2020).

Discoloration observation on growth rate of *M. hyopneumoniae* strains

The test strains were incubated in a humidified incubator at 37℃ for 72 h, negative control of the culture medium was also set up. The discoloration was observed every 12 h until the color of culture medium changed from red to bright yellow. Every time point was set to have three independent replicates.

Determination of titer of *M. hyopneumoniae* strains

The titers of the *M. hyopneumoniae* strains were quantified every 12 h until 72 h using the 50% color change unit (CCU_{50}) assay (Leigh et al., 2008), which was modified from the CCU assay with no contamination by other pathogens. Every timepoint was set three independent replicates.

Metabolite extraction

Metabolism-free extraction on two representative pig breeds (Large white pig and Bama miniature pig) with six samples from each breed, were performed. Methanol aqueous solution(400 μL of 80%) was added into an Rnase-free 2 mL tube, and then vortexed followed by ice bath for 5 min. The solution was centrifuged at 15,000 g

for 20 min at 4℃, the supernatant was diluted with mass spectrometry grade water to 53% methanol, and centrifuged at 15,000 g for 20 min at 4℃. The supernatant was collected and injected into LC–MS for analysis (Beier et al., 2018; Want et al., 2006).

UHPLC-MS/MS analysis

Ultra-high performance liquid chromatography-MS/MS (UHPLC-MS/MS) analysis was performed using a Vanquish UHPLC system (ThermoFisher, Germany) coupled with an Orbitrap Q Exactive^{TM}HF-X mass spectrometer (ThermoFisher, Germany) in Novogene Co., Ltd. (Beijing, China). The detailed procedure was according to a previous study (Wu et al., 2020) with some modifications. Briefly, samples were injected into a Hypesil Gold column (100 × 2.1 mm, 1.9 μm) using a 17-min linear gradient at a flow rate of 0.2 mL/min. The eluents for the positive polarity mode were eluent A (0.1% FA in Water) and eluent B (Methanol). The eluents for the negative polarity mode were eluent A (5 mM ammonium acetate, pH 9.0) and eluent B (Methanol). The solvent gradient was set as follows: 2% B, 1.5 min; 2–100% B, 3 min; 100% B, 10 min; 100–2% B, 10.1 min; 2% B, 12 min. Q Exactive™ HF-X mass spectrometer was operated in positive/negative polarity mode with a spray voltage of 3.5 kV, capillary temperature of 320 °C, sheath gas flow rate of 35 psi and aux gas flow rate of 10 L/min, S-lens RF level of 60, Aux gas heater temperature of 350 °C.

Data processing and metabolite identification and analysis

The raw data files generated by UHPLC-MS/MS were processed using the Compound Discoverer 3.1 (CD3.1, ThermoFisher) to perform peak alignment, peak picking, and quantitation for each metabolite. The main parameters were set as follows: retention time tolerance, 0.2 min; actual mass tolerance, 5 ppm; signal intensity tolerance, 30%; signal/noise ratio, 3; and minimum intensity, et al. After that, peak intensities were normalized to the total spectral intensity. The normalized data was used to predict the molecular formula based on additive ions, molecular ion peaks and fragment ions. Then peaks were matched with the mzCloud (https://www.mzcloud.org/), mzVault and MassList database to obtain the accurate qualitative and relative quantitative results. Statistical analyses were performed using the statistical software R (R version R-3.4.3), Python (Python 2.7.6 version) and CentOS (CentOS release 6.6), when data were not normally distributed, normal transformations were attempted using area normalization method. These metabolites were annotated using the KEGG database (https://www.genome.jp/kegg/pathway.html), HMDB database (https://hmdb.ca/ metabolites) and LIPID Maps database (http://www.lipidmaps.org/). Principal components analysis (PCA) and Partial least squares discriminant analysis (PLS-DA) were performed at Meta X (Wen et al., 2017). Univariate analysis (t-test) was applied to calculate the statistical significance (*P*-value). The metabolites with VIP > 1 and *P*-value < 0.05 and fold change ≥ 2 or FC ≤ 0.5 were considered to be differential metabolites. Volcano plots were used to filter metabolites of interest which based on log2 (Fold Change) and -log10(*p*-value) of metabolites by ggplot2 in R language. Correlation between differential metabolites were analyzed by correlation analysis function cor () in R language (method = Pearson). *P*-value < 0.05 was considered as statistically significant, and statistical significance was expressed through cor.mtest () in the R language. Functions of these metabolites and metabolic pathways were studied using the KEGG database. The metabolic pathways enrichment of differential metabolites was performed, when ratio was satisfied by x/n > y/N, metabolic pathway was considered as enrichment. When *P*-value of metabolic pathway < 0.05, metabolic pathways were considered as statistically significant enrichment.

Preparation medium with the addition of representative local and introduced pig breed serum and L-Asparagine

L-Asparagine (L-Asn) (Cat. No A4159-100 mg, Sigma-Aldrich, Shanghai, China) was prepared using KM2 serum-free medium to 20 μM, 50 μM, 100 μM, 200 μM and 400 μM L-Asn, KM2 serum-free medium without L-Asn adding was used as control, and then added with non-hemolytic representative pig serum (20% (v/v)) from local pig breed Bama miniature pigs and introduced pig breed Large white pigs, after filtered through 0.22 μm filters, respectively. Serum samples were collected from three individuals of Bama miniature pig and Large white pig species.

Determination of pH for growth changes of *M. hyopneumoniae* strains

The test strains were placed in a humidified incubator at 37 °C for 72 h, negative control of the culture medium was also set up. In conjunction with the discoloration of strains, pH value of the bacterial solution was determined every 12 h as well.

Quantitative real-time PCR assay of *M. hyopneumoniae*

A 20 μL sample of each strain culture medium was taken at 12-h intervals and frozen at -70 ℃. After 72 h, all samples were collected at a total of 7 time points and then tested for real-time PCR. The real-time PCR reaction system (20 μL) was composed as follows: 10 μL AceQ qPCR probe Master Mix (Vazyme Biotech Co., Ltd), 2 μL template, 1 μL forward primers, 1 μL reverse primer, 1 μL probes, 5 μL ddH_2O. Each run included a positive control (the gradient dilution of recombinant plasmid), a

negative control (ddH2O). The reaction conditions were as follows: 40 cycles of 50 °C for 2 min, 95 °C for 10 min; 95 °C for 15 s, 60 °C for 60 s). The reaction was carried out in Quant Studio 5 Real-Time PCR System (Applied Biosystems, Foster City, CA, USA). Templates were tested in triplicate and the Cycle threshold (CT) values were plotted against the copy number in order to verify the reproducibility. Quantification of copy number of *M. hyopneumoniae* by TaqMan quantitative PCR for further analysis of its metabolic capacity (Wu et al., 2019). The samples were run in triplicate biological replicates.

Adherence ability of *M. hyopneumoniae* in medium supplemented with Bama miniature pig serum and Large white pig serum with/without L-Asn

Green fluorescent labelled cell proliferation tracer CFDA-SE (Cat. No C1031, Beyotime, Shanghai, China) was used to label the *M. hyopneumoniae* strains LH and J, which were cultured in medium supplemented with 20% Bama miniature pig serum and Large white pig serum, respectively, named B-LH and B-J where medium added Bama miniature pig serum ± 400 μM L-Asn, and W-LH and W-J where culture medium added Large white pig serum ± 400 μM L-Asn for 30 min, before infected with immortalized porcine broncholiar epithelial cells (hTERT-PBECs) (Xie et al., 2018) for 12 h. Detailed procedure of adhesion test of *M. hyopneumoniae* to cells according to our previous study with some modifications (Xie et al., 2022). Briefly, after hTERT-PBECs were fixed with 4% paraformaldehyde, treated with 0.1% Triton X-100, blocked for 2 h in 3% (w/v) BSA in PBS, cells were stained with the mouse monoclonal antibody (anti-P97R1, made in our lab) at a dilution of 1:400. Next, actin protein was labeled by iFluor™ 555 phalloidin (Cat. No 40737ES75, YEASEN, Shanghai, China) for 1 h before cell visualization was conducted after staining the nuclei with DAPI (Cat. No C1005, Beyotime, Shanghai, China) for 2 min at RT with a fluorescence microscope (Zeiss, Tokyo, Japan).

In addition, two *M. hyopneumoniae* strains (LH (high-virulence) and J (low-virulence) cultured in medium ± L-Asn supplemented with 20% Bama miniature pig and Large white pig serum named W-LH, B-LH, W-J, B-J, were selected for adhesion rate determination, After 12 h infection, cells were then digested with 0.25% trypsin (without EDTA), and concentration of strain before and after infection was calculated with the CCU_{50} test. Before inoculation, the DMEM/F12 cell culture medium was diluted 10 times after centrifugation for 20 min at 11,000 r/min to ensure that each strain was approximately set to 10^7 CCU_{50}/mL in three independent replicates.

Cytotoxicity before and after infection with *M. hyopneumoniae* cultured in medium supplemented with Bama mininature and Large white pig serum alone or with/ without adding L-Asn

HTERT-PBECs were incubated for 6 h and grown to confluence in 24-well plates. After three washes with PBS, the cells were then incubated with four *M. hyopneumoniae* strains (strains LH, 168, J and 168L, titers of which were 1×10^8 CCU_{50}/mL) cultured with no serum KM2 medium supplemented with 20% Large white pig or Bama miniature pig serum alone, or adding 0, 50 and 400 μM L-Asn in the no-serum KM2 medium supplemented with the above two pig serum. In addition, cells infected with 20% LPS (5 μg/mL), DMSO (1% diluted in DMEM/F12 medium) and infected with *M. hyopneumoniae* strains were used as positive controls, cells without treatement were used as negative control. All groups were performed with three independent replicates. After 6 h, culture supernatants were measured for LDH activity using a CytoTox 96® Non-Radioactive Cytotoxicity Assay (Cat No. G1780, Promega, Madison, WI, USA) according to the manufacturer's instructions (Hao et al., 2022).

ELISA validation for serum metabolic biomarkers

All randomly selected six serum and BALF samples from Bama miniature pigs and Large white pigs before and after infection with *M. hyopneumoniae* were used for ELISA assays to detect the concentrations of three serum metabolic biomarkers, L-Asn, Inositol (Ino) and Arachidonic acid (LCT4) according to the instructions of the kits including L-Asparagine (Cat. No YX-E28661H), Inositol (Cat. No YX-E28726H), Arachidonic acid (Cat. No YX-E28660H), which were all purchased from Sinobestbio Shanghai, China.

Statistical analysis

All experiments were reproducible, carried out in triplicate, and all data were expressed as mean ± SD. Differences in the growth titers of *M. hyopneumoniae* strains under different test conditions, changes in copy number per 10 μL of *M. hyopneumoniae* strains, comparison of the concentrations of biomarkers from serum and BALF samples of pigs of two species before and after infection, cytotoxicity assay of hTERT-PBECs after *M. hyopneumoniae* infection cultured in medium supplemented with Bama miniature pig and Large white pig serum alone, with or without adding L-Asn were assessed through one-way analysis of variance (ANOVA) followed by Tukey's multiple-comparison test for post hoc analysis using SPSS Statics v20.0 and GraphPad Prism 8.3 software. $P < 0.05$ was considered

a significant difference, and $P < 0.01$ was considered an extremely significant difference.

Results and discussion

Discoloration time and growth titers of *M. hyopneumoniae* supplemented with Chinese pig breeds serum were much earlier and with higher titer compared to those in introduced pig breeds serum

As shown in Table 1, after 14 days of in vitro culture in medium supplemented with Chinese pig breeds serum demonstrated higher titer compared to those in introduced pig breed serum. Among them, especially cultured with Bama miniature pig serum resulted in faster growth rates and significant increment in CCU_{50} titers by approximately 0.25–0.5 log compared to the serum from Large white pigs and Duroc pigs. This effect was more pronounced in highly virulent *M. hyopneumoniae* strains (168, LH) compared to less virulent strains (168L, J).

Similarly, as shown in Figure S1, compared with introduced pig breed, *M. hyopneumoniae* cultured in medium supplemented with representative local pig breed Bama miniature pig serum, particularly during the first 7 days, not only the color change occurred 12 to 24 h earlier. In addition, growth titers of *M. hyopneumoniae* strains were also much higher compared to those in introduced pig breeds serum no matter strains differed in virulence. Consistent with our previous study, demonstrating that pathogenic degree of lung lesions caused by *M. hyopneumoniae* infection in local pig breeds Bama miniature pig was more severe than that in introduced pig breeds Large white pig and Landrace, and local pig breeds exhibited higher morbidity and more severe symptoms, while imported breeds such as Large white pigs and Landrace showed relatively lower susceptibility to *M. hyopneumoniae* (Gan et al., 2020). In this study, Chinese pig breeds such as Bama miniature pigs and Meishan pigs had significantly better cultivation results for *M. hyopneumoniae* in porcine serum-based media compared to imported pig breeds like Large white pigs and Duroc. The growth rate and titers of both highly virulent and low-virulent strains in the Bama miniature pig and Meishan pig serum groups were significantly higher than those in the Large white pigs and Duroc serum groups. Considering that porcine serum is an important source of nutrients, speculating that there may be certain differing substances in the serum between different pig breeds, which could be crucial factors facilitating the successful infection of *M. hyopneumoniae* in Chinese pig breeds.

Metabolomic analysis

With the development of various omics technologies such as genomics, proteomics, and metabolomics, the analysis of *Mycoplasma* genomes and protein functions through high-throughput genomic sequencing, protein screening, and metabolite analysis has provided more data and experimental basis for studying the metabolism of these minimal microorganisms (Kamminga et al., 2017; Wang et al., 2018, 2020). Among them, metabolomics has been used to identify dormancy and complex interactions between hosts and pathogens (Newsom & McCall, 2018; Patti et al., 2012). Metabolic events that occur during host–pathogen interactions reflect the host's response to the pathogen and how the pathogen adapts and proliferates in the host environment (Newsom & McCall, 2018). The application of metabolomics in studies of human and animal infectious diseases have revealed new knowledge about the biochemical and physiological processes of viral, bacterial, and parasitic infections (Denery et al., 2010; Shin et al., 2011; Voge et al., 2016), and can provide important guide the identification of diagnostic biomarkers.

In this study, untargeted metabolomic analysis of serum from representative introduced pig breed, Large white pigs (W), and representative local pig breed, Bama miniature pigs (B), metabolites and their changes were identified and analyzed. The metabolomic results were subjected to visualization and analysis, as shown in Fig. 1-A, significant separation appeared in the PCA

Table 1 Comparison of titers (CCU_{50}/mL) of *M. hyopneumoniae* strains supplemented with different pig breeds serum under static culture conditions

M.hyopneumoniae strains	Large white pigs	Duroc pigs	Meishan pigs	Bama miniature pigs
168	7.75 ± 0.25 *	7.75 ± 0.50 *	8.00 ± 0.25	8.50 ± 0.125
168L	8.25 ± 0.25 *	8.25 ± 0.50 *	8.50 ± 0.25	8.75 ± 0.25
LH	8.50 ± 0.25 *	8.25 ± 0.50 *	8.50 ± 0.50	9.00 ± 0.125
J	8.25 ± 0.50 *	8.50 ± 0.25 *	8.75 ± 0.50	8.75 ± 0.25
NJ	8.25 ± 0.25 *	8.25 ± 0.50 *	8.50 ± 0.25	8.75 ± 0.125

No significant difference of titers compared between different *M.hyopneumoniae* strains in each pig breed shown in each colum. For each *M.hyopneumoniae* strain, titers of strain cultured with medium supplemented with Bama miniature pig serum were significantly higher than those supplemented with Large white pig serum and Duroc pig serum shown in each row. Data reflect the means ± standard deviation (SD) of three independent experiments. *: $p < 0.05$ indicates that the difference is significant

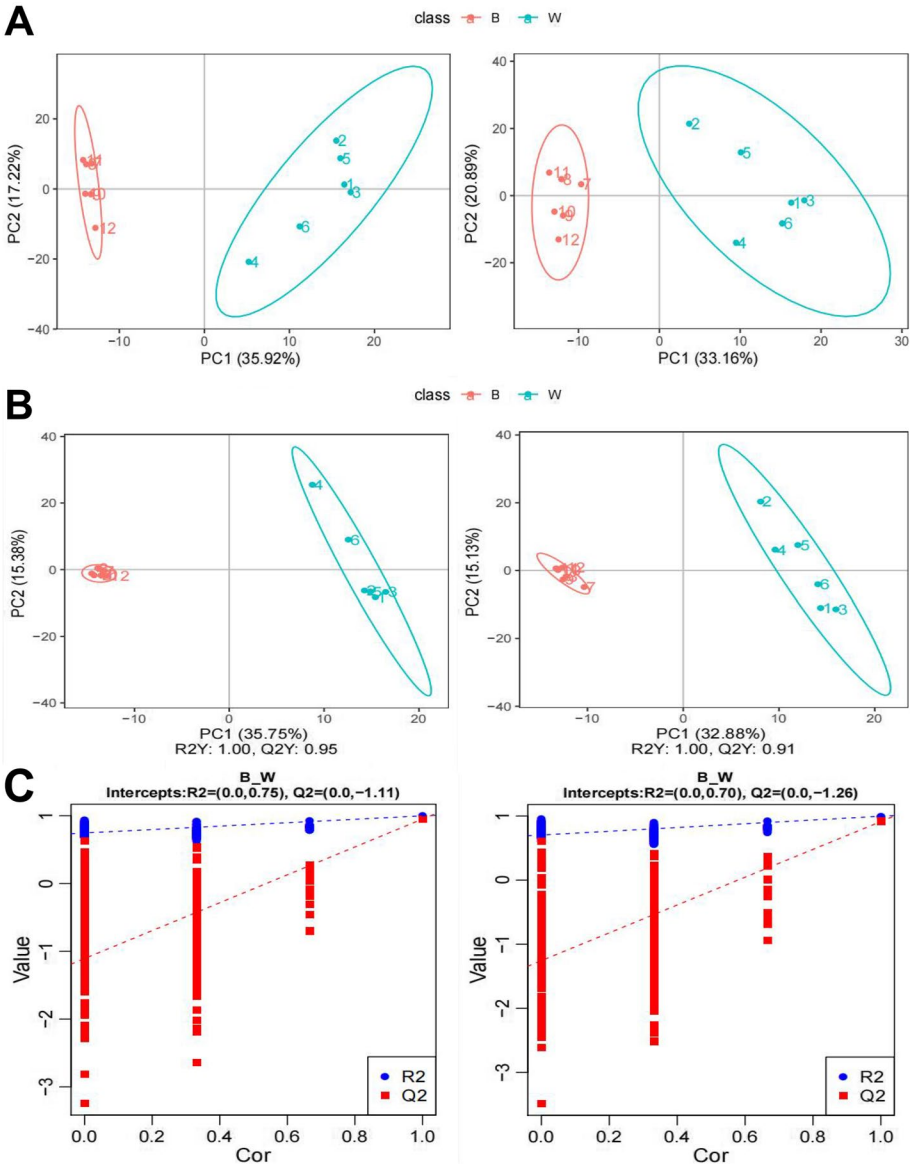


Fig. 1 Untargeted metabolomic analysis of representative introduced and local pig breeds Large white pigs and the Bama miniature pigs. **A** Principal Component Analysis (PCA) (left panel for positive ion mode, right panel for negative ion mode): The x-axis represents the scores of the first principal component (PC1), and the y-axis represents the scores of the second principal component (PC2). The different colored dots represent samples from different experimental groups, and the ellipses indicate the 95% confidence interval. **B** Partial least squares discriminant analysis (PLS-DA) score plot (left panel for positive ion mode, right panel for negative ion mode): The x-axis represents the scores of samples on the first principal component, and the y-axis represents the scores on the second principal component. R2Y represents the model's explained variation, and Q2Y is used to evaluate the predictive ability of the PLS-DA model. When R2Y is greater than Q2Y, indicating a well-established model. **C** Validation plot (left panel for positive ion mode, right panel for negative ion mode): The x-axis represents the correlation between randomly grouped Y and original grouped Y, and the y-axis represents the scores of R2 and Q2

plot, indicating good clustering of samples within each group. PLS-DA analysis was performed on two groups of metabolites, results showed R2Y > Q2Y, indicating it is a well-established model (Fig. 1-B). The predictive ability was further validated using permutation tests. After 200 permutations, the regression line intercepts of the R2 and Q2 values obtained from positive and negative ionization modes were as shown in Fig. 1-C. R2 values were 0.75 and 0.70 in positive and negative ionization modes, respectively, while the Q2 regression line intercepts were -1.11 and -1.26 in positive and negative ionization modes, respectively. These results indicated that the PLS-DA

model was well-fitted, subsequent data analysis was reliable and results were trustworthy.

Differential metabolite screening and identification

One thousand one hundred fourteen metabolites were detected, including 589 positive ions and 525 negative ions. Total number of significantly different metabolites was 428, including 235 positive ions and 193 negative ions. Among them, 163 metabolites showed significant upregulation, including 107 positive ions and 56 negative ions. A volcano plot was generated to display the differences in metabolite abundances based on the *P*-values obtained from t-tests, VIP values calculated by PLS-DA, and fold change (FC) values of metabolite concentrations (Figs. 2-A and C). Different metabolites coordinate their biological functions, and pathway analysis helps further understand their biological functions. Identified metabolites were subjected to GO functional and KEGG pathway annotation (Figs. 2-B and D), indicating that amino acid metabolism pathways were the major biochemical metabolic pathways in serum of both pig breeds.

Correlation analysis of differential metabolites revealed that the content of L-Asn in serum of Bama miniature pigs was 15–18 times higher than that in serum of Large white pigs, and it can be used as an indispensable nitrogen source for *M. hyopneumoniae*. L-Asn, a non-essential amino acid and an important precursor for various biological molecules, serves as a significant source of carbon, nitrogen, and energy in metabolic processes (Bogatyreva et al., 2006). *Mycobacterium tuberculosis* captures L-Asn using membrane transport proteins, which serves as an important additional nitrogen source during host colonization. This L-Asn uptake system supports the growth of the bacteria under acidic conditions through ammonia release and pH buffering (Gouzy et al., 2014). Furthermore, L-Asn accounted for 7.6% of the

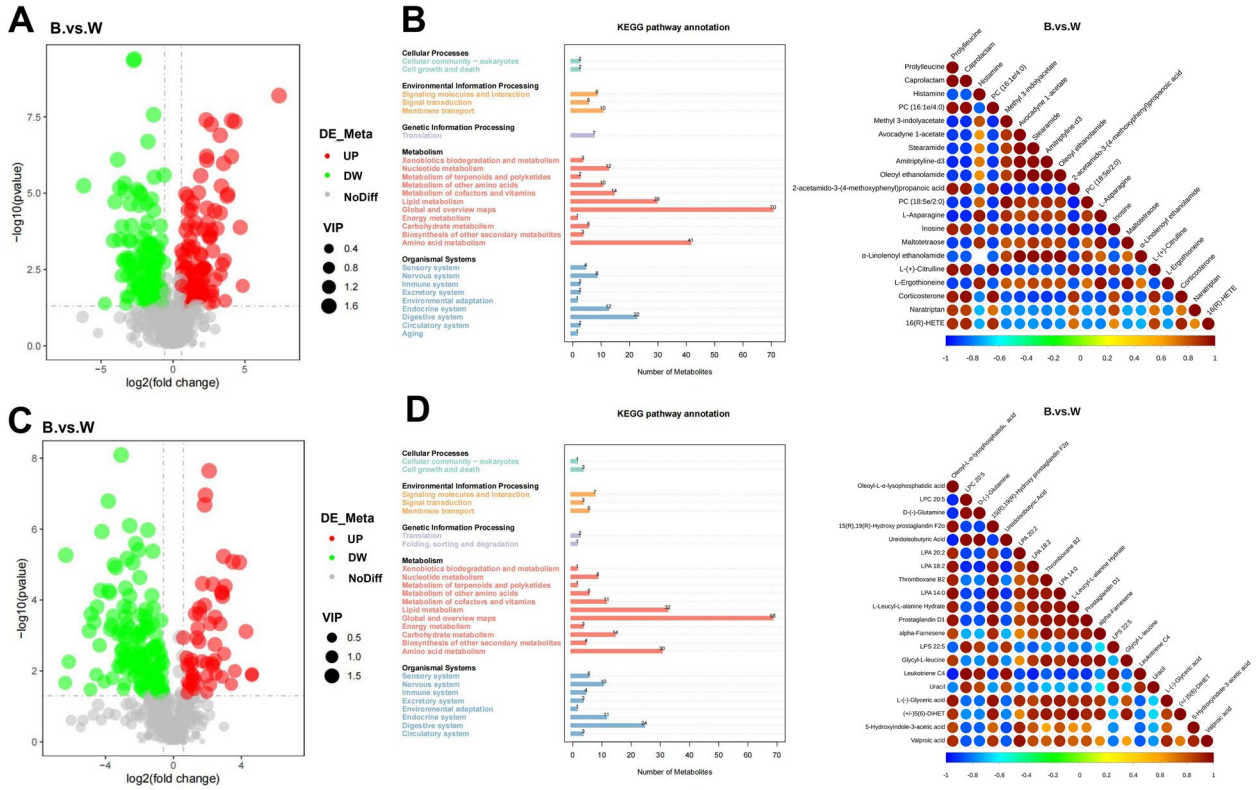


Fig. 2 Differentiation and identification of non-targeted serum metabolites from Large white pig and Bama miniature pig samples, respectively. **A** and **C** Volcano plots of differential metabolites (A for positive ion mode, C for negative ion mode): The x-axis represents the log2-fold change of metabolites between different groups, and the y-axis represents the significance level (-log10p-value). Each point in the volcano plot represents a metabolite, with significantly upregulated metabolites shown in red and significantly downregulated metabolites shown in green. The size of the dots represents the VIP value. **B** and **D** KEGG functional annotation and correlation analysis of metabolites (B for positive ion mode, D for negative ion mode): KEGG pathway annotation: The x-axis represents the number of metabolites, and the y-axis represents the annotated KEGG pathways, showing the number of metabolites annotated in each second-level category under the first-level classification of pathways. Correlation analysis of differential metabolites: The highest correlation value is 1, indicating a complete positive correlation (red), while the lowest correlation value is -1, indicating a complete negative correlation (blue). The parts without color indicate *P*-value > 0.05. The graph displays the correlation of the top 20 differential metabolites sorted in ascending order of *P*-value

protein composition in *M. hyopneumoniae*, a proportion significantly higher than the average protein composition in prokaryotes (4–5%) (Bogatyreva et al., 2006; Nagaraj et al., 2015), indicating that L-Asn is an important component of *M. hyopneumoniae* proteins. Through comparative genomic analysis, approximately 40% of *M. hyopneumoniae* strains (both virulent and non-virulent strains) lack L-Asn synthetase, an enzyme responsible for the synthesis of L-Asn from L-aspartic acid. These strains cannot synthesize L-Asn internally and should only rely on exogenous L-Asn to meet their protein synthesis needs. In mammals, L-Asn enhances host LCK signaling to enhance CD8(+) T cell activation and host antitumor response, and depletion of L-Asn by L-asparaginase can be used as a target drug for the treatment of tumor cells (Wu et al., 2021). In parasites such as *Plasmodium berghei*, L-Asn is critical for the development of its asexual and hepatic stages, as well as for host infection (Yadav & Swati, 2012). In bacteria, reducing L-Asn concentrations prevents the growth of group A *streptococci* in blood and mouse models of bacterial infection (Baruch et al., 2014). In viruses, L-Asn is able to affect viral protein synthesis and is a limiting factor in the replication of *cowpox virus* and *human cytomegalovirus*, and has been regarded as a candidate target for broad-spectrum antiviral drugs (Lee et al., 2019; Pant et al., 2019). All of the above findings suggest that L-Asn plays an important role in tumorigenesis as well as in the proliferation and infection of parasites, bacteria, and viruses.

Additionally, substances such as inositol and arachidonic acid were also significantly upregulated in serum of Bama miniature pigs. For inositol a cyclic sugar alcohol with important biological functions that are widely present in nature, participating in various substance metabolism activities in animal and plant cells, and it was considered to be as a biologically essential active substance for microorganism growth. Moreover, it has diverse biological functions and plays a significant role in cell signal transduction, intracellular transport, and osmotic regulation (Chatree et al., 2020). Arachidonic acid (LCT4), an unsaturated fatty acid released through the hydrolysis of membrane phospholipids by phospholipases, serves as a precursor for inflammatory reactions and directly regulates the activity of various enzymes, ion channels, and the generation of bioactive substances, playing a significant regulatory role in the physiological functions and pathological processes of cells and organisms (Seeds & Bass, 1999). To systematically understand whether L-Asn plays a key role in the growth and pathogenesis of *M. hyopneumoniae*, and make it clear whether it was related to susceptibility of Chinese pig breeds to *M. hyopneumoniae*, comprehensive studies were conducted.

Bama miniature pig serum and a certain concentration of L-Asn could promote the growth of viable bacteria of *M. hyopneumoniae* strains

As shown in Figure S2, the growth rate of *M. hyopneumoniae* in group B was significantly faster than that in group W in terms of color change, regardless of virulence of *M. hyopneumoniae* strains. The growth rate of *M. hyopneumoniae* strains in both groups supplemented with L-Asn accelerated significantly, although there are some differences existed in two groups. In general, strains in group W need to add with a high concentration of L-Asn, whereas the group B needs the addition of a low concentration of L-Asn e. According to the growth pH change curves of each *M. hyopneumoniae* strain (Figs. 3A-a and A-b), time period with the greatest pH decrease was selected (with the pH value between 6.5 to 6.8) for the following *M. hyopneumoniae* growth titer detection. *M. hyopneumoniae* strains NJ and 168 had the greatest pH decrease between 48 and 60 h (pH between 6.5 and 6.8), and LH, J, and 168L had a pH decrease between 0 and 48 h that was more pronounced and leveled off after 48 h.

Moreover, certain concentrations of L-Asn could significantly promote the growth of *M. hyopneumoniae*. Highly virulent strains NJ (Fig. 3B-b) and 168 (Fig. 3B-c) required the addition of higher concentrations of L-Asn, while strains cultured in group B required much lower concentrations of L-Asn than those in group W, only with some slight difference because of strain difference. Low virulent strains J (Fig. 3B-d) and 168L (Fig. 3B-e) required lower L-Asn concentrations. LH (Fig. 3B-a), unlike other *M. hyopneumoniae* strains, the growth of LH is more dependent on exogenous L-Asn due to its lack of asparagine synthetize, thus, LH showed a greater increase in growth titers with the addition of L-Asn than other *M. hyopneumoniae* strains, consistently with a lower requirement for L-Asn in the B group than those in group W. Compared with titers of the original *M. hyopneumoniae* strains that were also passaged once without any additives, in the presence of 400 μM L-Asn, strain LH showed an increase in titer of 0.50(W) and 0.58(B); strain NJ showed an increase in titer of 0.42(W) and 0.50(B); strain 168 showed an increase in titer of 0.42(W) and 0.50(B); in the presence of 50 μM L-Asn, strain J showed an increase in titer of 0.42(W) and 0.50(B); 168L strain showed an increase in titer of 0.50(W) and 0.50(B).

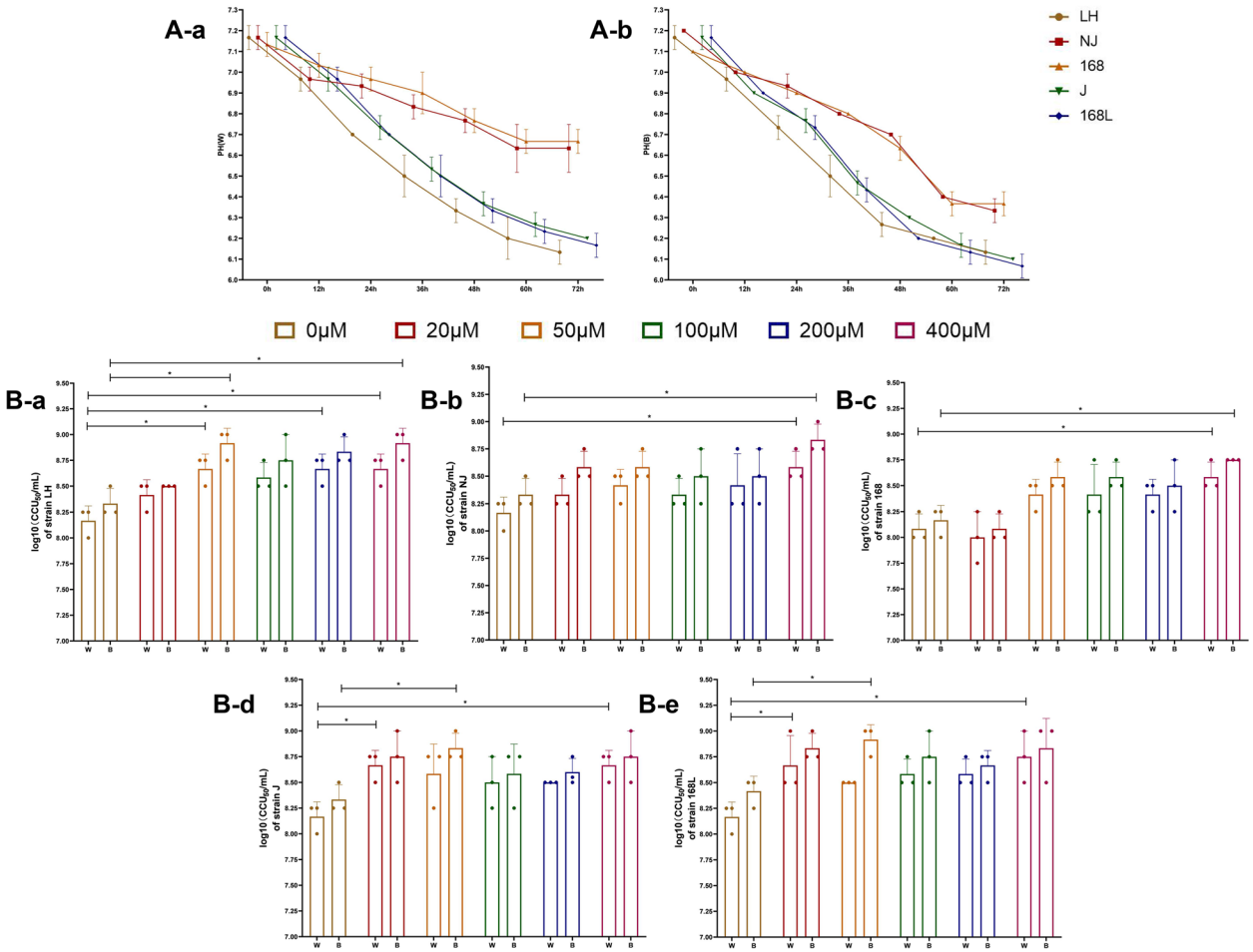


Fig. 3 Determination of pH values of different *M. hyopneumoniae* strains cultured in vitro in the serum medium from Large white pigs and Bama miniature pigs, as well as viable titers of *M. hyopneumoniae* strains cultured in the serum medium from Large white pigs and Bama miniature pigs plus different concentrations of L-Asn. **A**-a pH growth curve of different *M. hyopneumoniae* strains in serum medium of Large white pigs. **A**-b pH growth curve of different *M. hyopneumoniae* strains in serum medium of Bama miniature pigs. **B**-a Effect of different concentrations of L-Asn on the growth titer of *M. hyopneumoniae* strain LH. **B**-b Effect of different concentrations of L-Asn on the growth titer of *M. hyopneumoniae* strain NJ. **B**-c Effect of different concentrations of L-Asn on the growth titer of *M. hyopneumoniae* strain 168. **B**-d Effect of different concentrations of L-Asn on the growth titer of *M. hyopneumoniae* strain J. **B**-e Effect of different concentrations of L-Asn on the growth titer of *M. hyopneumoniae* strain168L. *: $P < 0.05$, indicating a significant difference

A certain concentration of L-Asn could promote the metabolic capacity of *M. hyopneumoniae* strains

Similar to the *M. hyopneumoniae* growth titers, the DNA copy numbers of *M. hyopneumoniae* in group B were higher than those in group W, and the metabolic capacity assay of *M. hyopneumoniae* strains supplemented with L-Asn, was shown in Fig. 4. Highly virulent strains LH, NJ and 168 had much more concentration requirement for L-Asn than lowly virulent strains J and 168L.

Bama miniature pig serum and L-Asn could promote the adhesion and cytotoxicity of *M. hyopneumoniae*

The adhesion of all microorganisms, including *Mycoplasma*, to their host cells is a key step for their colonization and subsequent infection of the host. Adhesion ability is an important factor that reflects bacterial virulence (Wu et al., 2019; Zhang et al., 2021). *M. hyopneumoniae* is mainly found on the mucosal surface along the entire swine respiratory tract, including the trachea, bronchi and bronchioles, inducing ciliostasis and loss of cilia. Thus, the first stage of pathogenesis is the adhesion of *M. hyopneumoniae* to the cilia of the epithelial cells of the respiratory mucosa by means of adhesions. Adhesion and colonization of host respiratory mucosal epithelial cells by *M. hyopneumoniae* is one of the key factors for successful infection, and the pathogenic mechanism of *M. hyopneumoniae* is mainly through adherence to respiratory epithelial cells, thereby destroying the mucosal

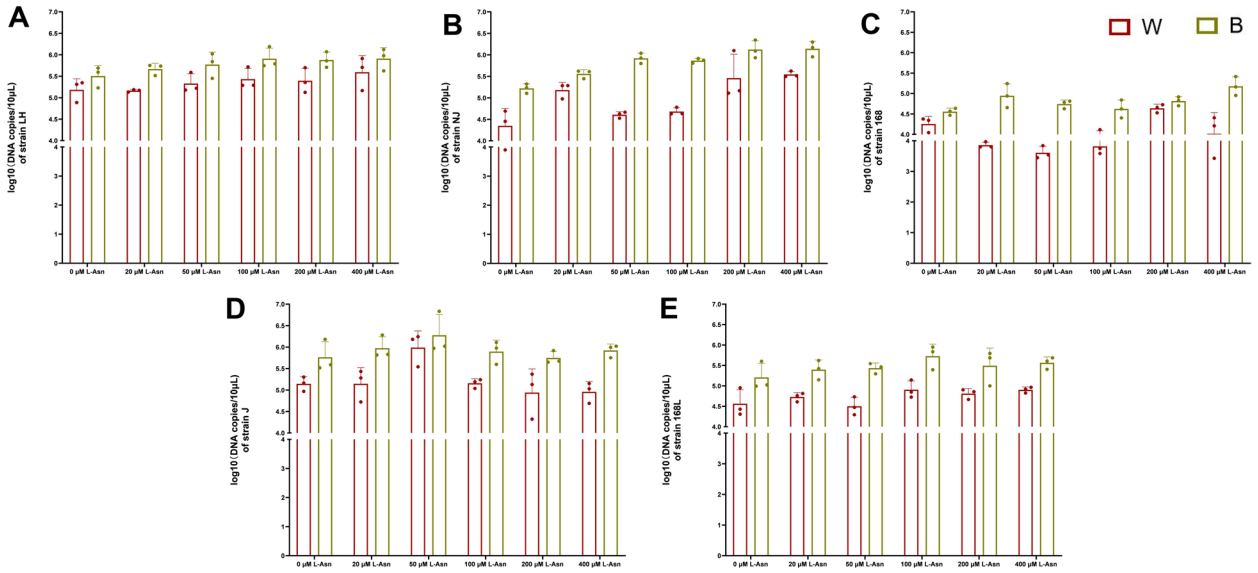


Fig. 4 Effects of different concentrations of L-Asn on the metabolic capacity of *M. hyopneumoniae* strains cultured in vitro in the serum medium of Large white pigs and Bama miniature pigs. **A** Effects of different concentrations of L-Asn on the metabolic capacity of *M. hyopneumoniae* strain LH. **B** Effects of different concentrations of L-Asn on the metabolic capacity of *M. hyopneumoniae* strain NJ. **C** Effects of different concentrations of L-Asn on the metabolic capacity of *M. hyopneumoniae* strain 168. **D** Effects of different concentrations of L-Asn on the metabolic capacity of *M. hyopneumoniae* strain J. **E** Effects of different concentrations of L-Asn on the metabolic capacity of *M. hyopneumoniae* strain 168L

epithelium and causing inflammatory reactions (Kumar, 2018). *M. hyopneumoniae* adheres to host cells while extending microtubules into the cells at the adhesive end, releasing hydrogen peroxide and superoxide free radicals. These substances interact with endogenous toxic oxygen molecules produced by host cells, which can cause oxidative stress in the respiratory epithelium, leading to complete shedding of epithelial cilia, swelling, dissolution, and necrosis of cells. Here, adhesion ability and adhesion rate of *M. hyopneumoniae* strains cultured in culture medium with serum in group B was significantly stronger than those in medium with serum in group W (Fig. 5A and C). A significant difference existed of *M. hyopneumoniae* strain LH between group B with or without 400 μM L-Asn ($p < 0.05$), the addition of 400 μM L-Asn to the serum of group W significantly enhanced *M. hyopneumoniae* adhesion ability and adhesion rate irrespective of the virulence of strains LH and J (Fig. 5B and D).

In addition, superoxide free radicals can inhibit cellular catalase, further exacerbating the damage (Waites & Talkington, 2004). The interaction of these substances with endogenous toxic oxygen molecules produced by host cells can cause oxidative stress in the respiratory epithelium. In this study, cytotoxicity of *M. hyopneumoniae* cultured in the serum group B was significantly higher than that of serum group W (Fig. 6A) to hTERT-PBECs, significant differences existed between *M. hyopneumoniae* strain NJ and 168 cultured with medium plus serum of group W ($p < 0.05$), and cytotoxicity in serum group B induced by *M. hyopneumoniae* LH, 168, J were all significantly higher than that in *M. hyopneumoniae* 168L ($p < 0.01$), cytotoxicity of *M. hyopneumoniae* 168 in group B was also significantly higher than that in group W ($p < 0.01$). Meanwhile, the addition of L-Asn at different concentrations to the serum medium of group W (Fig. 6B) and group B (Fig. 6C) both significantly increased the cytotoxicity induced by *M. hyopneumoniae* strains. Moreover, cytotoxicity triggered by the addition of L-Asn to the serum medium of group B was more severe, especially at high concentrations of L-Asn in both pig breeds serum. Specifically, for *M. hyopneumoniae* strains in group W by addition of L-Asn with different concentrations (Fig. 6B), cytotoxicity of *M. hyopneumoniae* 168 in group W adding 50 μM L-Asn was also significantly higher than that in group W without L-Asn adding ($p < 0.05$), while when *M. hyopneumoniae* strains cultured in group W adding with 400 μM L-Asn, cytotoxicity induced by *M. hyopneumoniae* strain LH ($p < 0.05$), 168($p < 0.01$), J ($p < 0.05$) and 168L ($p < 0.05$) were significantly higher than those in group W without adding L-Asn. Similarly, for *M. hyopneumoniae* strains in group B by addition of L-Asn with different concentrations (Fig. 6C), cytotoxicity induced by *M. hyopneumoniae* increased with the increment of concentration of L-Asn, especially when strains cultured in group B adding with 400 μM L-Asn, cytotoxicity induced by strain

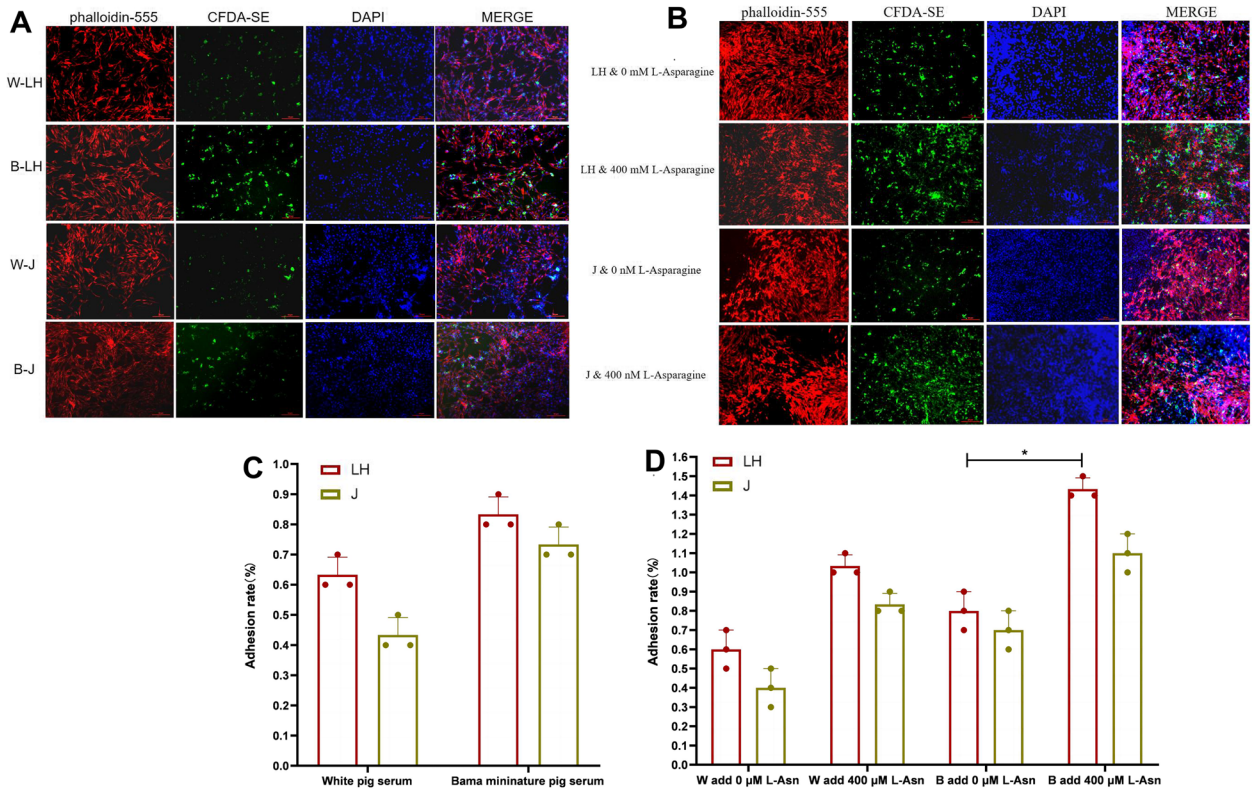


Fig. 5 Effects of different concentrations of L-Asn and serum of two representative pig breeds on the cell adhesion ability to *M. hyopneumoniae*. **A** and **C** Effect of *M. hyopneumoniae* on the adhesion ability of hTERT-PBECs in the serum medium of Large white pigs and Bama miniature pigs. **B** and **D** Effects of *M. hyopneumoniae* strains on the adhesion ability of hTERT-PBECs in the serum medium of Large white pigs and Bama miniature pigs with or without different concentrations of L-Asn. *: $P < 0.05$, indicating a significant difference

LH ($p < 0.05$), 168 ($p < 0.05$), J ($p < 0.05$) and 168L ($p < 0.05$) were all significantly higher than those in group W without adding L-Asn.

Concentration of L-Asn, Ino and LCT4 were significantly higher in clinical serum and BALF samples after *M. hyopneumoniae* infection

As shown in Fig. 7, the concentrations of L-Asn, Ino and LCT4 were all higher in the serum and BALF samples in group B than in group W, which was consistent with the results of the non-targeted metabolism; all three metabolic biomarkers showed extreme significant differences ($p < 0.01$) when compared between pre-infection W serum and pre-infection B serum, or between post-infection W serum and post-infection B serum. In addition, no matter in group W or group B, concentrations of all three biomarkers no matter tested in serum or BALF post-infection are higher compared to those before *M. hyopneumoniae* infection, although with no significant difference. Moreover, concentrations of L-Asn, Ino and LCT4 in serum and BALF were significantly higher before *M. hyopneumoniae* infection compared with those after *M. hyopneumoniae* infection, indicating that these three metabolites were consumed during *M. hyopneumoniae* infection, and more were consumed in group B, which further strengthened the conclusion that L-Asn is indeed related to the growth metabolism and pathogenicity of *M. hyopneumoniae*.

Although currently no research has been reported about L-Asn in *Mycoplasmas* infection, a few studies have shown that AsnRS, a key protein in the L-Asn utilization pathway, plays a role in the formation of the biofilm of *Mycoplasma*. By comparing the planktonic and biological characteristics of *Mycoplasma* fermentans and *Mycoplasma pneumoniae*, AsnRS was found to be critical for the growth and survival of *Mycoplasma* and is an essential gene for *Mycoplasma* survival (Awadh et al., 2021). Detailed mechanism of L-Asn involved in *M. hyopneumoniae* infection merits being further studied in the future and this is also our future research direction.

Conclusion

In this study, through non-targeted metabolomic analysis of serum from representative introduced and Chinese pig breeds, Large white pigs and Bama miniature pigs, three significantly up-regulated metabolites, namely, L-Asn,

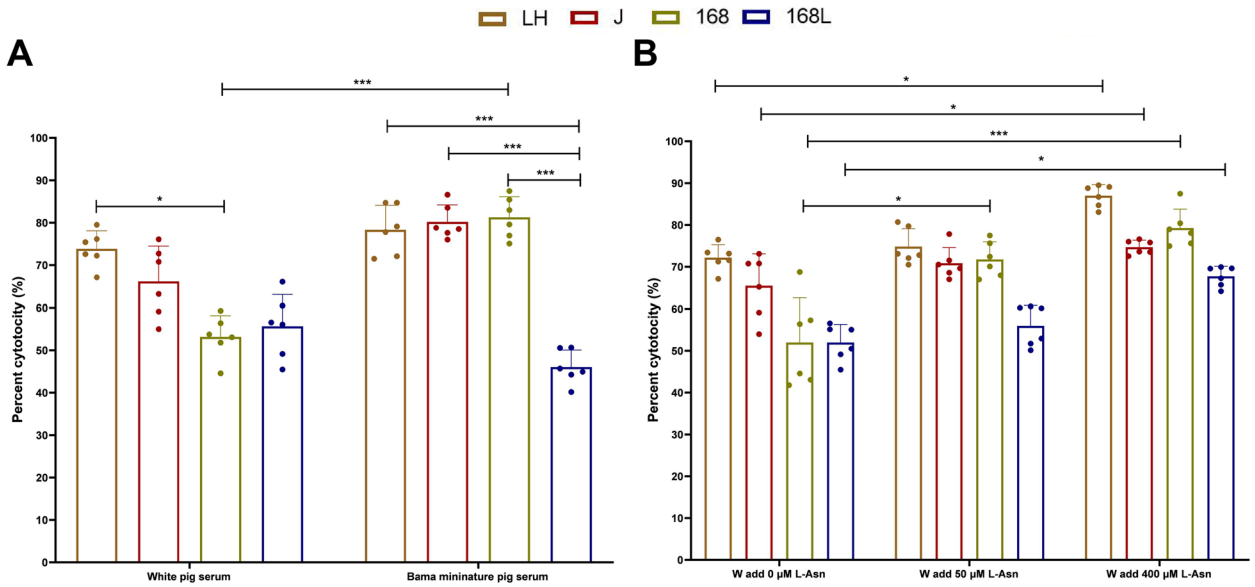


Fig. 6 Effect of different concentrations of L-Asn and serum from two representative pig breeds on the cytotoxicity on hTERT-PBECs induced by different *M. hyopneumoniae* strains. **A** Comparison of cytotoxicity of different *M. hyopneumoniae* strains cultured in vitro in serum medium of Large white pigs and Bama miniature pigs.b **B** Comparison of cytotoxicity of different *M. hyopneumoniae* strains by adding different concentrations of L-Asn to the serum medium of Large white pigs. **C** Comparison of cytotoxicity of different *M. hyopneumoniae* strains by adding different concentrations of L-Asn to the serum medium of Bama miniature pigs. ***: $P < 0.01$, indicating an extremely significant difference; *: $P < 0.05$, indicating a significant difference

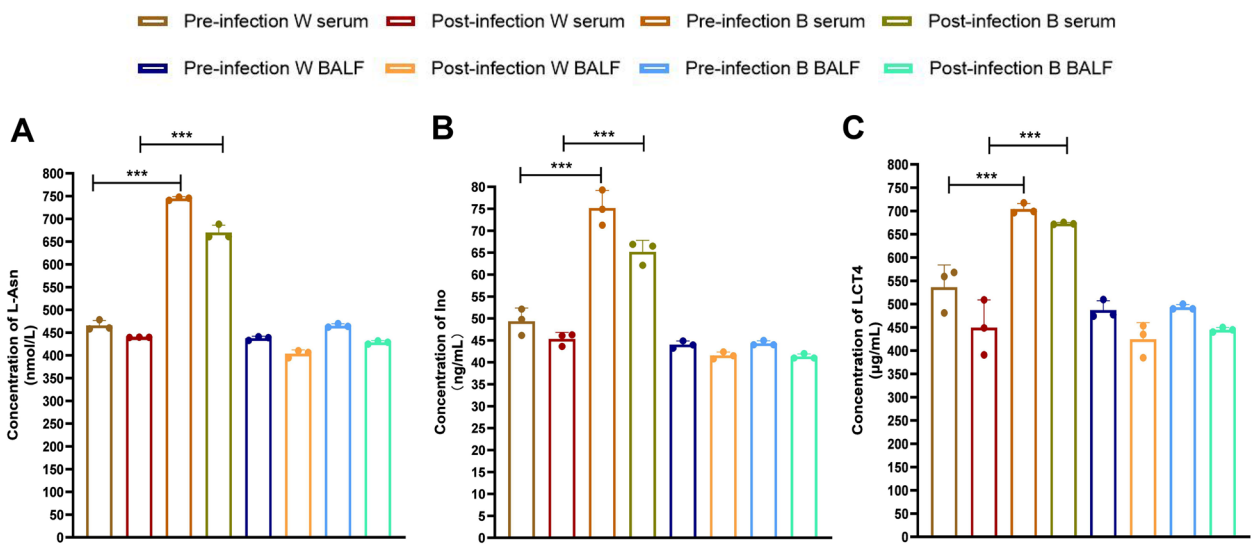


Fig. 7 Concentration of three metabolic biomarkers in serum and BALF before and after infection of *M. hyopneumoniae* in Large white pigs and Bama miniature pigs detected by ELISA. **A** Concentration of L-Asn in serum and BALF of Large white pigs and Bama miniature pigs before and after *M. hyopneumoniae* infection. **B** Concentration of Ino in serum and BALF of Large white pigs and Bama miniature pigs before and after *M. hyopneumoniae* infection. **C** Concentration of LCT4 in serum and BALF of Large white pigs and Bama miniature pigs before and after *M. hyopneumoniae* infection. ***: $P < 0.01$, indicating an extremely significant difference

Ino and LCT4 were screened, speculating that these metabolites may be key factors in the susceptibility to *M. hyopneumoniae* infection of local pig breeds in China, and L-Asn, the most significant different serum metabolic marker, the effects of which on the growth, metabolism capability and pathogenicity of *M. hyopneumoniae*,

was clarified. 400 μM and 50 μM L-Asn could promote the growth and metabolism of *M. hyopneumoniae*, and 400 μM L-Asn could promote the adhesion and cytotoxicity of *M. hyopneumoniae*. Our present study could partially explain the reasons that local pig breeds in China are more susceptible to *M. hyopneumoniae*, which will offer a theoretical basis for preventing and controlling the occurrence, optimize the culture medium of *M. hyopneumoniae*, and will promote the healthy development of the pig industry eventually.

Abbreviations

ANOVA	One-way analysis of variance
BALF	Bronchoalveolar lavage fluid
B	Bama miniature pig
W	Large white pig
CSFV	Classical swine fever virus
EP	European Pharmacopoeia
HMDB	Human Metabolome Database
Ino	Inositol
KEGG	Kyoto encyclopedia of genes and genomes
L-Asn	L-asparagine
LCT4	Arachidonic acid
M. hyopneumoniae	Mycoplasma hyopneumoniae
MPS	Mycoplasma pneumonia of swine
PRRSV	Porcine respiratory and reproductive syndrome virus
PCV2	Porcine circovirus type 2
PCA	Principal components analysis
PLS-DA	Partial least squares discriminant analysis
RT	Room temperature
SF-pCD	Snatch-farrowed porcine-colostrum-deprived
BALF	Bronchoalveolar lavage fluid
UHPLC-MS/MS	Ultra-high performance liquid chromatography-MS/MS
hTERT-PBECs	Immortalized porcine broncholiar epithelial cells
SD	Standard deviations

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s43014-024-00282-0.

Supplementary Material 1.

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Authors' contributions
LZhao conducted most of the experiments and wrote the manuscript; FH helped with ELISA, CCU_{50} experiment, cell adhesion and cytotoxicity tests; LZhang helped with pig sample collection; YNW helped *M. hyopneumoniae* strains culture; QYX, RC, YFY helped to analyze the data; ZXF & XX conceived this study and contributed in the design as well as coordination. All authors read, agreed and approved the final manuscript.

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Data availability
Data and materials obtained and analyzed in this study are shown in this manuscript and supplementary file.

Declarations

Ethics approval and consent to participate
Animal experiments were approved by the Committee on the Ethics of Animal Experiments (Protocol # PDC 2023003) and were performed in strict accordance with the animal regulations of Jiangsu Province (Government Decree No. 45) at Jiangsu Academy of Agricultural Sciences (License No. SYXK (Su) 2020–0023).

Consent for publication
Not applicable.

Competing interests
The authors declare that they have no conflict of interest.

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