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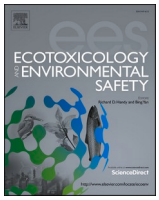

Construction of recombinant *Pichia pastoris* strains for ammonia reduction by the *gdh*A and *gln*A regulatory genes in laying hens

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ABSTRACT

Ammonia emissions have become an important environmental challenge for the livestock industry. Probiotics are often used as additives to reduce ammonia, and the ammonia reduction efficiency of common probiotics is approximately 20–40%. In this study, we constructed a *gdh*A recombinant *Pichia pastoris* strain, *gln*A recombinant *Pichia pastoris* strain and *gdh*A-*gln*A *Pichia pastoris* recombinant strain using the *gdh*A and *gln*A genes, which have the potential function of reducing ammonia emissions. The results of *in vitro* fermentation showed that compared with the control, wild-type *Pichia pastoris* and pPICZA strains, the *gdh*A, *gln*A and *gdh*A-*gln*A recombinant strains significantly reduced ammonia emissions in laying hens ($P < 0.05$), with emission reduction efficiencies of 63.95%, 65.68% and 74.04%, respectively. The reason may be that the recombinant *Pichia pastoris* strains can convert ammonium nitrogen into amino acids for self-growth through ammonia assimilation, and reduce the pH, uric acid and urea content in the intestinal tract of livestock and poultry, and urease activity. Therefore, the construction of recombinant strains can provide technical support for reducing ammonia pollution in the livestock industry.

1. Introduction

Odor pollution caused by the livestock industry has become an increasingly serious social and environmental problem. The composition of the odor is complex, where ammonia (NH_3) is the predominant noxious gas emitted from farms (Feilberg et al., 2010). The emission of NH_3 decreases air quality, thus affecting the health and welfare of humans and livestock (Hofmann et al., 2020). NH_3 production leads to a large amount of atmospheric nitrogen settling on the earth's surface, causing water eutrophication and soil acidification (Aneja and Schlesinger, 2008). High concentrations of NH_3 reduce the production performance of livestock and poultry but also induce respiratory diseases, pulmonary edema and other diseases, which are severe diseases that can lead to death (Stokstad, 2014; Guiziou and Beline, 2005; Deaton et al., 1982). Working in a high-ammonia-concentration environment for a long time stimulates the skin, eyes and soft tissue (Schiffman and S, 1998). Therefore, reducing NH_3 emissions is currently one of the key topics associated with the sustainable development of the livestock industry and improvement of the quality of life of rural residents.

The main methods commonly used to reduce the odor of livestock and poultry are the regulation of the intestinal microbes of livestock and poultry, including adjusting the composition of diets, using feed additives and probiotics (Pan et al., 2016; Yang et al., 2020; Gibson et al., 2004). Among them, probiotics, because of their pollution-free nature, and lack of residue, are widely considered for application in the livestock odor reduction (Zommiti et al., 2020). Currently, the most widely used probiotics in the livestock and poultry industry include lactic acid bacteria, *Bacillus* species, yeast (Alagawany et al., 2018). It has been reported that *B. subtilis* C-1302 supplementation at 600 mg/kg of feed can reduce NH_3 production by approximately 20% in broilers (Jeong and Kim, 2014). Jiandui et al. (2019) found that *Pichia farinosa, Bacillus coagulans, Lactobacillus plantarum, Pichia guilliermondii*, and *Bacillus subtilis* reduced NH_3 production by approximately 35.1–39% during *in vitro* fermentation of cecal contents of laying hens. The NH_3 reduction effect of most probiotics still needs to be improved.

Probiotics can be modified through genetic engineering technology

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to improve their NH_3 emission reduction efficiency. Many studies have shown that bacteria can convert inorganic nitrogen such as ammonium nitrogen into organic nitrogen through ammonia assimilation (ter Schure et al., 1995; Sieg and Trotter, 2014), which is of great significance for reducing NH_3 emissions. In our previous studies, *Enterococcus faecium* strain and *Bacillus coagulans* strain were screened from laying chicken caecal and faecal samples which have good ammonia-degrading abilities. Under the condition of ammonia-nitrite stress, the expression levels of the *gdh*A gene of *Enterococcus faecium* strain and the GMPS gene and *gln*A gene of *Bacillus coagulans* strain increased significantly. On this basis, the recombinant *E. coli* strain was constructed for *in vitro* fermentation test verification, results showed that *gdh*A and *gln*A may be key genes involved in the bacterial-mediated regulation of NH_3 emissions by laying hens, and ammonia emissions may be reduced by regulating their expression (Xiao et al., 2021). Glutamate dehydrogenase (GDH) encoded by the *gdh*A gene and glutamine synthetase (GS) encoded by the *gln*A gene are the key enzymes involved in ammonia assimilation (Ps et al., 2019). Magasanik's (Magasanik, 2003) research showed that GDH in yeast can transfer NH_3 to 2-ketoglutarate to form glutamic acid. Transferring the GSI gene from conifers to poplars improve the nitrogen use efficiency and increased the production of glutamine in poplars (Gallardo et al., 1999). The results of these studies all indicate that the GDH and GS encoded by the *gdh*A and *gln*A genes have the potential to reduce ammonium nitrogen in the environment, thereby reducing NH_3 emissions. Therefore, *gdh*A gene and *gln*A gene can be introduced into probiotics to construct recombinant strains, thereby improving the NH_3 emission reduction effect of probiotics.

Pichia pastoris (*P. pastoris*) can be widely used in poultry and animal production as a probiotic (Damasceno et al., 2012). Moreover, *P. pastoris* is also a widely used system for heterologous protein expression (Schwarzhans et al., 2017). In recent years, methylotrophic yeast *P. pastoris* has been developed as a highly successful system for the production of a variety of heterologous proteins. Some of its key advantages include the achievement of high cell densities, high expression of heterologous proteins, processing and modification of the expression product, and maintenance of its activity (Kumar and Mutturi, 2020). Gil et al. (2012) reported that recombinant *P. pastoris* containing the Clostridium perfringens alpha toxin gene can be used as a probiotic for broilers. Chen et al. (2000) found that feeding broiler chickens *P. pastoris* and recombinant *P. pastoris* expressing swine growth hormone (rGH) can improve the growth performance of broilers.

Therefore, in the present study, *P. pastoris* was selected as the receptor cell to introduce the *gdh*A and *gln*A genes, which are the key genes for ammonia assimilation, and induce the expression of GDH and GS. The effect of NH_3 reduction was verified by an *in vitro* fermentation experiment. In this study, a recombinant strain that can efficiently reduce NH_3 emissions was constructed, which provides a theoretical basis and evidence for further application in the livestock industry.

2. Materials and methods

2.1. Ethics statement

The present study followed the institutional guidelines for the care and use of animals, and all experimental procedures involving animals were approved by the Animal Experimental Committee of South China Agricultural University (Ethics Approval Code: SYXK 2014–0136).

2.2. Microorganism materials

P. pastoris GS115 and the vector pPICZA were purchased from Invitrogen (Carlsbad, CA, USA). *Escherichia coli* DH5α, *Enterococcus faecium strain* and *Bacillus coagulans strain* were preserved in the Livestock Ecology Laboratory, South China Agricultural University. *Enterococcus faecium strain* and *Bacillus coagulans strain* were screened from the cecum of laying hens.

2.3. Gene primers design and the expression vector construction

Primers were designed according to the sequences of the *gdh*A (Text S1) and *gln*A (Text S1) genes. As described in Table 1, the *gdh*A gene was amplified using the primers GDH (F) and GDH (R) with *Xho*I and *Not*I restriction sites, and the *gln*A gene was amplified using the primers GS (F) and GS (R) with *Kpn*I and *Not*I restriction sites.

2.4. PCR amplification and sequencing

The *gdh*A gene in the *Enterococcus faecium strain* and the *gln*A gene in the *Bacillus coagulans strain* were used as templates, the recombinant vector pPICZA-*gdh*A was used with GDH(F) and GDH(R) as primers to synthesize the *gdh*A gene sequence, and the recombinant vector pPICZA-*gln*A was used with GS(F) and GS(R) as primers to synthesize the *gln*A gene sequence. The PCR system was as follows: 0.5 μL of TaKaRa Ex Taq, 5 μL of 10 ×Ex Taq Buffer, 4 μL of dNTP mixture, 1 μL of DNA template, 1 μL of forward primer, 1 μL of reverse primer, and 37.5 μL of double-distilled water. PCR was conducted as follows: 95 °C for 3 min; 30 cycles of 95 °C for 30 s, annealing at 55 °C for 90 s, and 72 °C for 1 min; and 72 °C for 10 min. The PCR products were identified and imaged by 1.0% agarose gel electrophoresis. Another sample was sent to Shanghai Songon Biotech Co., Ltd., for sequencing. The primers are listed in Table 1.

2.5. DNA manipulation and transformation

The vector pPICZA and the *gdh*A gene fragment were digested by the enzymes *Xho*I and *Not*I, and the reaction system was as follows: 20 μL of gene, 5 μL of 10 × Buffer K, 2.5 μL of *Xho*I, 2.5 μL of *Not*I, and 20 μL of double-distilled water. The vector pPICZA and the *gln*A gene fragment were successfully digested by the enzymes *Kpn*I and *Not*I, and the reaction system was as follows: 20 μL of gene, 5 μL of 10 × Buffer K, 2.5 μL of *Kpn*I, 2.5 μL of *Not*I, and 20 μL of double-distilled water. The reaction system was mixed, centrifuged, and incubated overnight at 37 °C.

For gene knock-in, the target fragments were digested with restriction endonucleases and ligated to the expression vector pPICZA to obtain the corresponding plasmids. The reaction system was as follows: 1 μL of 10 ×T4 ligase buffer, 6 μL of target fragment, 2 μL of vector, and 1 μL of T4 ligase. The reaction system was mixed, centrifuged, and incubated overnight at 16 °C, and the ligation product was used for the next linearization process and then used for transformation. Furthermore, genomic integration was carried out by linearization of the plasmid using *Pme*I, and the reaction system was as follows: 1 μL of plasmid, 5 μL of buffer, 1 μL of *Pme*I, and 24 μL of double-distilled water. The product was then transformed into *P. pastoris* GS115 competent cells according to the instructions for the *Pichia pastoris* positive clone assay kit (SK2430, Coolaber, Beijing, China), and the cells were selected using medium containing 100 μg/mL zeocin. The DNA of the recombinant *P. pastoris* strains was extracted and identified by PCR. The plasmids of the recombinant *P. pastoris* strains were extracted and sent to Shanghai Songon Biotech Co., Ltd., for sequencing. The primers used are universal primers for pPICZA.

Table 1
Primers used in the study.

Gene	Primer name	Primer sequence
*gdh*A	GDH (F)	CCGCTCGAGATGACAAACGCAACAGAATATGTT
	GDH (R)	ATAAGAATGCGGCCGCAACTAAACCTTCGCTG
*gln*A	GS (F)	GGGGTACCATGCCTAAGTTTACACGTGAAGATATTTTGAAGA
	GS (R)	ATAAGAATGCGGCCGCATACATTTCGATATACTGGTCGCGTTCCC

The underlined texts are the restriction sites.

2.6. Microbial culture conditions

These recombinant *P. pastoris* strains were inoculated into YPDA liquid medium containing zeocin and cultured at 30 °C and 200 rpm for 24 h. Next, a 1% inoculum volume was added to 500-mL flasks containing 50 mL of BMGY medium (2% peptone, 1% yeast extract, 1% glycerol, 1.18% KH_2PO_4, 0.223% K_2HPO_4, 1.34% yeast nitrogen base [YNB], and 0.04 μg/L biotin) and cultured at 30 °C and 200 rpm until the OD_{600} was 2–6. Then, the yeast cells were harvested by centrifugation at 5000 ×g for 10 min, cultured in 500-mL flasks containing BMMY medium (2% peptone, 1% yeast extract, 1% methanol, 1.18% KH_2PO_4, 0.223% K_2HPO_4, 1.34% YNB, and 0.04 μg/L biotin) and cultured at 30 °C and 200 rpm for 144 h. To compensate for methanol volatilization, 1% methanol was added every 24 h. Samples were collected every 24 h and placed in a refrigerator at − 80 °C for enzyme activity detection. Total protein was extracted for western blot analysis. GDH was extracted and measured using a Glutamate Dehydrogenase (GDH) Assay Kit (BC1460, Solarbio, Beijing, China). GS was extracted and measured using a Glutamine Synthetase (GS) Assay Kit (BC0910, Solarbio, Beijing, China). The Minute™ Total Protein Extraction Kit for Microbes with Thick Cell Walls (Invent Biotechnologies, Plymouth, MN) was used to extract total protein for Western blot analysis. The pPICZA vector contained the sequences encoding a polyhistidine (6*His) tag in the C-terminus. Thus, the anti-His monoclonal antibody and goat polyclonal secondary antibody to mouse IgG were used in the Western blot assay for the recombinant protein.

2.7. Optimization of induced expression of recombinant P. pastoris strains

To further improve the enzyme productivity and achieve maximal bioactivity, the effects of culture volume, inoculum size, and methanol concentration on GDH and GS activity were investigated in shake-flask experiments based on the initial cultivation conditions (culture volume, 10%; inoculum size, 1%; methanol, 1%). All variables were fixed at a certain value, and only one variable was changed at a time (culture volume, 10%; inoculum size, 1%; methanol, 1%). A total of three values for each variable were used to optimize the induction of expression: culture volume, 5%, 10%, 15%; inoculum size, 1%, 2%, 3%; and methanol, 1%, 2%, 3%. At the end of the expression induction, samples were collected for the detection of enzyme activity.

2.8. Verification of the ammonium nitrogen removal effect of recombinant P. pastoris strains

Preliminary verification of the ammonium nitrogen removal effect of the recombinant strains was performed as follows: YNB was used as the basic medium, and glucose and ammonium sulfate were added exogenously to maintain a constant carbon-nitrogen ratio of the medium such that the ammonium nitrogen concentrations were approximately 0.1, 0.3, 0.6 and 1.2 mg/mL, the pH of the mediums were 5.8. Five strains, namely, wild-type *P. pastoris*, pPICZA, the *gdh*A, the *gln*A, and the *gdh*A-*gln*A recombinant strains, were cultivated under various ammonium nitrogen concentrations. Sampling was performed every 12 h to determine the concentration of ammonium nitrogen at the culture medium in different time periods to preliminarily determine the ammonium nitrogen utilization effect of recombinant *P. pastoris* strains.

2.9. In vitro fermentation

Sixty Lohmann Pink laying hens at 33 weeks of age were sacrificed to obtain cecal contents. The cecal contents were then vigorously mixed, diluted 1:3 (*w/v*) with buffer solution (35 g of $NaHCO_3$ plus 4 g of NH_4HCO_3 per L), and preheated at 42 °C. The cecal contents and buffer mixtures were individually squeezed through four layers of surgical gauze into the bottle and continuously bubbled with CO_2 at 42 °C.

In vitro gas production was measured as described by Menke and Steingass (1988), modified as applicable for research on chickens. Thirty-six 100 mL syringes (Häberle, Schwerte, Germany) were used for the *in vitro* gas production study. Each container was filled with fermentation inoculum (30 mL), 500 mg of the diet and different types of exogenous additives. The treatments were as follows: 500 mg of the diet plus 3 mL of double-distilled water (control), 500 mg of the diet plus 3 mL of wild-type *P. pastoris* (*P. pastoris*), 500 mg of the diet plus 3 mL of the pPICZA recombinant strain (pPICZA), 500 mg of the diet plus 3 mL of the *gdh*A recombinant strain (*gdh*A), 500 mg of the diet plus 3 mL of the *gln*A recombinant strain (*gln*A), and 500 mg of the diet plus 3 mL of the *gdh*A-*gln*A recombinant strain (*gdh*A-*gln*A). Each treatment group was replicated six times (syringes) and each syringe was used as an experimental unit. The strains used were the strain after induction of expression, and the conditions for inducing expression were as follows: the induced expression time was 5 days, culture volume was 10%; inoculum size was 2%; and methanol was 2%. After the induced expression, remove the components of the culture medium by centrifugation, then wash it with PBS solution, and finally suspend it with PBS solution and adjust it to the same OD_{600} value. Removing the air from the head-space, the syringe was sealed with a sealing clip, placed in a 42 °C (intestinal temperature of laying hens) incubator and rotated at 60 rpm for 12 h. After the end of the fermentation period, the syringes were placed in ice water to stop the continuous reaction. The gas accumulated in the headspace of each syringe was collected with a 50 mL gastight syringe (Hamilton, Reno, NV, USA) and the gas was immediately transferred into a bubble absorption tube containing sulfuric acid solution to collect NH_3. The fermentation broth in each syringe was transferred and centrifuged at 200g at 4 °C for 5 min. The supernatant was separated, transferred into another centrifuge tube, sealed, and then stored at −80 °C for determination pH, conductivity, ammonium nitrogen, nitrate nitrogen, urea, uric acid and urease activity.

2.10. Sample analysis

The NH_3 collected in the sulfuric acid solution was measured with a spectrophotometer based on the Chinese National Environmental Protection Standards (determination of ammonia nitrogen—Nessler's reagent spectrophotometry). A spectrophotometer was also used for the determination of nitrate nitrogen based on the Chinese National Environmental Protection Standards (determination of nitrate-nitrogen—spectrophotometric method with phenol disulfonic acid). A pH meter was used to determine the pH of the sample. The conductivity was measured with a conductivity meter. A uric acid assay kit (C012-1-1, NanJing JianCheng Bioengineering Institute, Nanjing, China) was used to determine the concentrations of uric acid. A urea assay kit (C013-1-1, NanJing JianCheng Bioengineering Institute, Nanjing, China) was used to determine the concentrations of urea. A soil urease (UE) test box (A121-1-1, NanJing JianCheng Bioengineering Institute, Nanjing, China) was used to determine the concentrations of uricase activities. The urease and uricase activities were determined using colorimetry according to Guan (Guan et al., 1986).

2.11. Statistical analyses

Statistical analysis was performed using software SPSS (version 22.0, Chicago, IL, USA). Normality and homogeneity of variances were verified by Shapiro–Wilk and Levene tests, respectively. The data were statistically analysed by one-way analysis of variance (ANOVA), and multiple comparisons were conducted by Duncan's test. For data with non-normality, nonparametric Kruskal–Wallis one-way ANOVA was used. Results were shown as mean ± standard error of the mean. Significance was accepted at $P < 0.05$.

3. Results

3.1. Construction of recombinant P. pastoris

In our previous studies, *Enterococcus faecium strain* and *Bacillus coagulans strain* screened from the cecum of laying hens have good ammonia-degrading abilities, and the *gdh*A and *gln*A genes were identified as being key in this process (Xiao et al., 2021). In this study, the *gdh*A recombinant strain, *gln*A recombinant strain, and *gdh*A-*gln*A recombinant strain were constructed through genetic engineering technology. Single colonies of the *gdh*A recombinant strain, *gln*A recombinant strain, and *gdh*A-*gln*A recombinant strain were cultivated, and the DNA of each strain was extracted. The extracted DNA was verified by polymerase chain reaction (PCR) (Fig. 1A) and was consistent with the target gene fragment, indicating that the *P. pastoris* expression system was successfully constructed. The plasmids sequencing results showed that the recombinant strains were correctly constructed. Total protein was extracted and verified by Western blotting with anti-His-tagged monoclonal antibodies. The results showed that the specific protein bands were approximately 50 and 55 kDa in molecular weight (Fig. 1B). The size was consistent with the expected size, indicating that the recombinant *P. pastoris* could correctly express the target protein.

3.2. Optimization conditions for expression in recombinant P. pastoris strains

Expression was induced in the recombinant *P. pastoris* strains under the conditions of 10% liquid volume, 1% inoculum volume and 1% methanol volume, and samples were collected every 24 h for enzyme activity detection. The relationship between induction time (in days) and the enzymatic activities of GS and GDH, which are expressed in *P. pastoris*, was examined (Fig. 2A). The GS activity of the wild-type *P. pastoris*, pPICZA and *gdh*A recombinant strains was almost unchanged during induced expression, while the GS activity of the *gln*A and *gdh*A-*gln*A recombinant strains varied as the number of days of induced expression increased significantly ($P < 0.05$), peaking on the 5th day. Fig. 2B shows the change in GDH activity under different induction times. The GDH activity of the wild-type *P. pastoris*, pPICZA and *gln*A recombinant strains was almost unchanged during induced expression, while the GDH activity of the *gdh*A and *gdh*A-*gln*A recombinant strains increased significantly with increasing time (days) of induced expression ($P < 0.05$), peaking on the 5th day.

All variables were fixed at a certain value, and only one variable was changed at a time (culture volume, 10%; inoculum size, 1%; methanol, 1%). A total of three conditions for each variable were tested to optimize the induction of expression: culture volume, 5%, 10% and 15%; inoculum size, 1%, 2% and 3%; and methanol 1%, 2%, and 3%. By detecting enzyme activity on the fifth day of expression induction, the appropriate conditions for inducing expression were screened. As shown in Fig. 3A-F, the maximum GS and GDH activities were obtained with a 10% culture volume, 2% inoculum size, and the daily addition of 2% (*v/v*) methanol to a shake-flask containing 10% culture medium.

3.3. Verification of the ammonium nitrogen removal effect of recombinant P. pastoris strains

When the initial ammonium nitrogen concentration of the culture medium was 0.1 mg/mL, the ammonium nitrogen removal rates of the *gdh*A-*gln*A, *gdh*A and *gln*A recombinant strains were 74.8%, 58.2% and 44.6%, respectively. The ammonium nitrogen removal rate of the recombinant strains was significantly higher than that of the wild-type *P. pastoris* and pPICZA strains, and the ammonium nitrogen removal rate of the double-gene recombinant strain was significantly higher than that of the single-gene recombinant strains ($P < 0.05$). However, as the initial ammonium nitrogen concentration increased, the ammonium nitrogen removal rate of the *gdh*A-*gln*A recombinant strain was lower than that of the other strains (Fig. 4).

3.4. Effect of recombinant P. pastoris strains on NH_3 production

In this study, odor gas emissions from laying hens were measured using an *in vitro* fermentation technique that has been reported as a reliable procedure for simulating gas production resulting from microbial fermentation in the cecum (Wang et al., 2016). During *in vitro* fermentation, the production of NH_3 in the *gdh*A, *gln*A and *gdh*A-*gln*A recombinant groups was significantly lower than that in the control, wild-type *P. pastoris* and pPICZA groups ($P < 0.05$), and the production

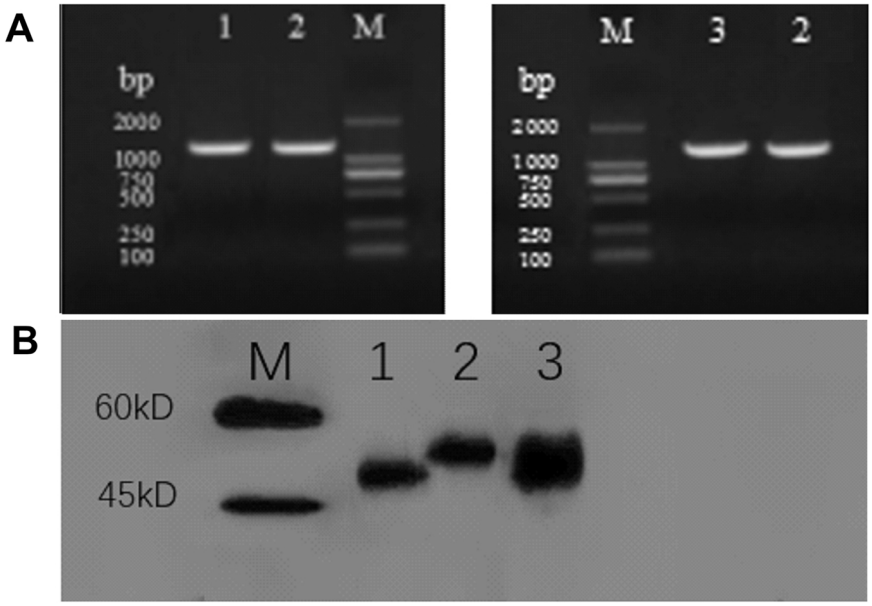

Fig. 1. A PCR verification of the recombinant strains. M: DL2000 marker; 1–2 are the *gdh*A genes of the *gdh*A and *gdh*A-*gln*A recombinant strains respectively; 2–3 are the *gln*A genes of the *gln*A and *gdh*A-*gln*A recombinant strains respectively. B Western blot of the target protein. M: protein molecular weight standard; 1–3 are the target proteins of the *gdh*A *gln*A and *gdh*A-*gln*A recombinant strains respectively.

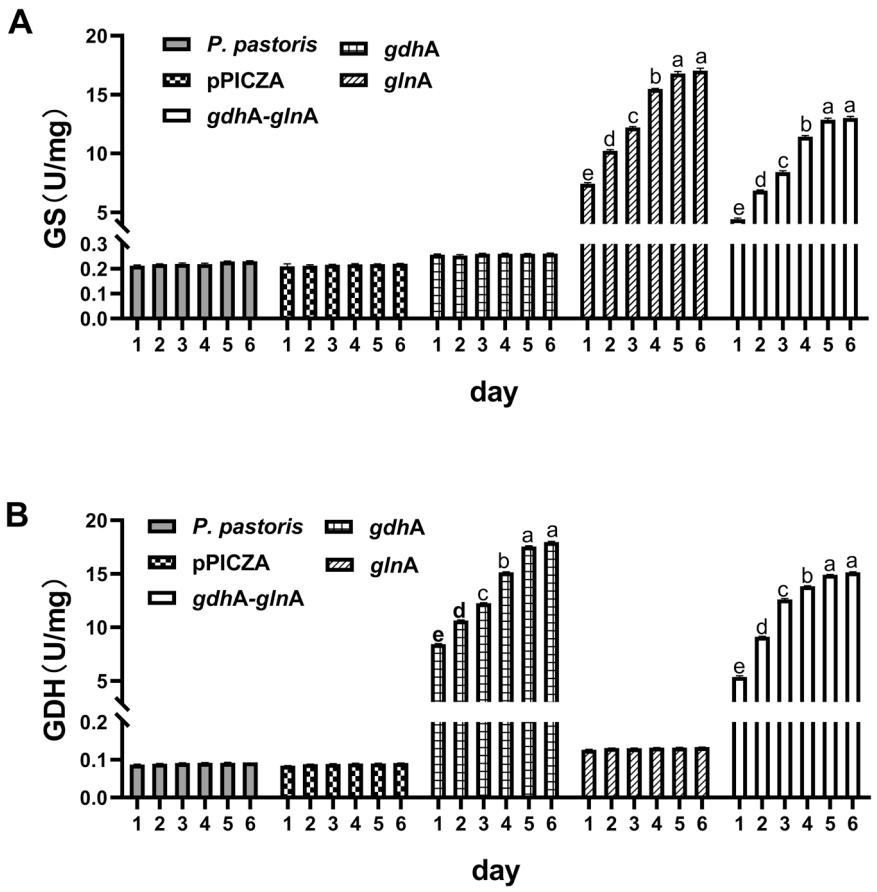

Fig. 2. Enzyme activity of the recombinant strain under different induction times. A Glutamate dehydrogenase activity of recombinant strains under different induction times. B Glutamine synthase activity of recombinant strains under different induction times. Error bars indicate standard error (n = 3). Different letters above the bars indicate statistically significant differences between the samples (ANOVA followed by Duncan's test, $P < 0.05$).

of NH_3 in the *gdh*A-*gln*A group was significantly lower than that in the other groups ($P < 0.05$). Compared with the control group, the average NH_3 production in the *P. pastoris*, pPICZA, *gdh*A, *gln*A and *gdh*A-*gln*A groups decreased by 37.58%, 38.44%, 63.95%, 65.68% and 74.04%, respectively (Fig. 5).

3.5. Effect of recombinant P. pastoris strains on biochemical cecal parameters

The effect of recombinant strains on biochemical cecal parameters is shown in Fig. 6. As shown in Fig. 6A-B, there was no significant difference in pH or the ammonium nitrogen content among the *gdh*A, *gln*A and *gdh*A-*gln*A groups ($P > 0.05$), but the levels in all these groups were significantly lower than those in the control, *P. pastoris*, and pPICZA groups ($P < 0.05$). This result indicated that the recombinant *P. pastoris* strain could significantly reduce the *in vitro* pH and the ammonium nitrogen content. The effect of recombinant *P. pastoris* strains on the nitrate nitrogen content is shown in Fig. 6C. There was no significant difference in the nitrate nitrogen content among the *gdh*A, *gln*A and *gdh*A-*gln*A groups ($P > 0.05$), but the levels in all these groups were significantly higher than those in the control, wild-type *P. pastoris* and pPICZA groups ($P < 0.05$).

The effects of recombinant *P. pastoris* strains on the levels of uric acid and urea are shown in Fig. 6D-E. The addition of recombinant *P. pastoris* strains showed significantly reduced levels of uric acid and urea. The difference was that the content of uric acid in the *gdh*A-*gln*A group was significantly lower than that in the *gdh*A and *gln*A groups ($P < 0.05$). As shown in Fig. 6F, the addition of recombinant *P. pastoris* strains yieled significantly reduced urease activity, and the urease activity of the *gdh*A-*gln*A group was significantly lower than that of the of the *gdh*A and *gln*A groups ($P < 0.05$).

4. Discussion

Based on probiotics, this study successfully constructed *gdh*A, *gln*A and *gdh*A-*gln*A recombinant strains through genetic engineering technology. Through the medium ammonia-degrading test and *in vitro* fermentation test, it was found that the introduction of the *gdh*A gene and *gln*A gene increased the NH_3 emission reduction efficiency of *P. pastoris* from approximately 35–75%. *P. pastoris*, as a probiotic, can effectively reduce the NH_3 emissions of laying hens (Jiandui et al., 2019), which is consistent with the results of this experiment. Compared with the wild-type *P. pastoris* and pPICZA groups, the addition of three types of recombinant *P. pastoris* strains can significantly reduce NH_3 emissions, and the NH_3 emission of the *gdh*A-*gln*A group was significantly lower than that of the *gdh*A and *gln*A groups ($P < 0.05$). These results indicate that the introduction of the *gdh*A and *gln*A genes can improve the NH_3 reduction effect of *P. pastoris*. The reason may be that GDH and GS reduce ammonium nitrogen in the cecum of laying hens through ammonia assimilation, thereby reducing NH_3 emissions. GDH and GS assimilate NH_3 through the GDH pathway and GS/GOGAT pathways, which are encoded by the *gdh*A and *gln*A genes, respectively (Meng et al., 2016). In microbes, both the GDH and GS/GOGAT (glutamate synthase) pathways play important roles in ammonia assimilation. GDH catalyze the incorporation of NH_3 into α-ketoglutarate to form glutamate directly (Helling, 1994); GS catalyze the amidation of glutamate to yield glutamine, and GOGAT catalyze the reductive transfer of the amide group from glutamine to α-ketoglutarate

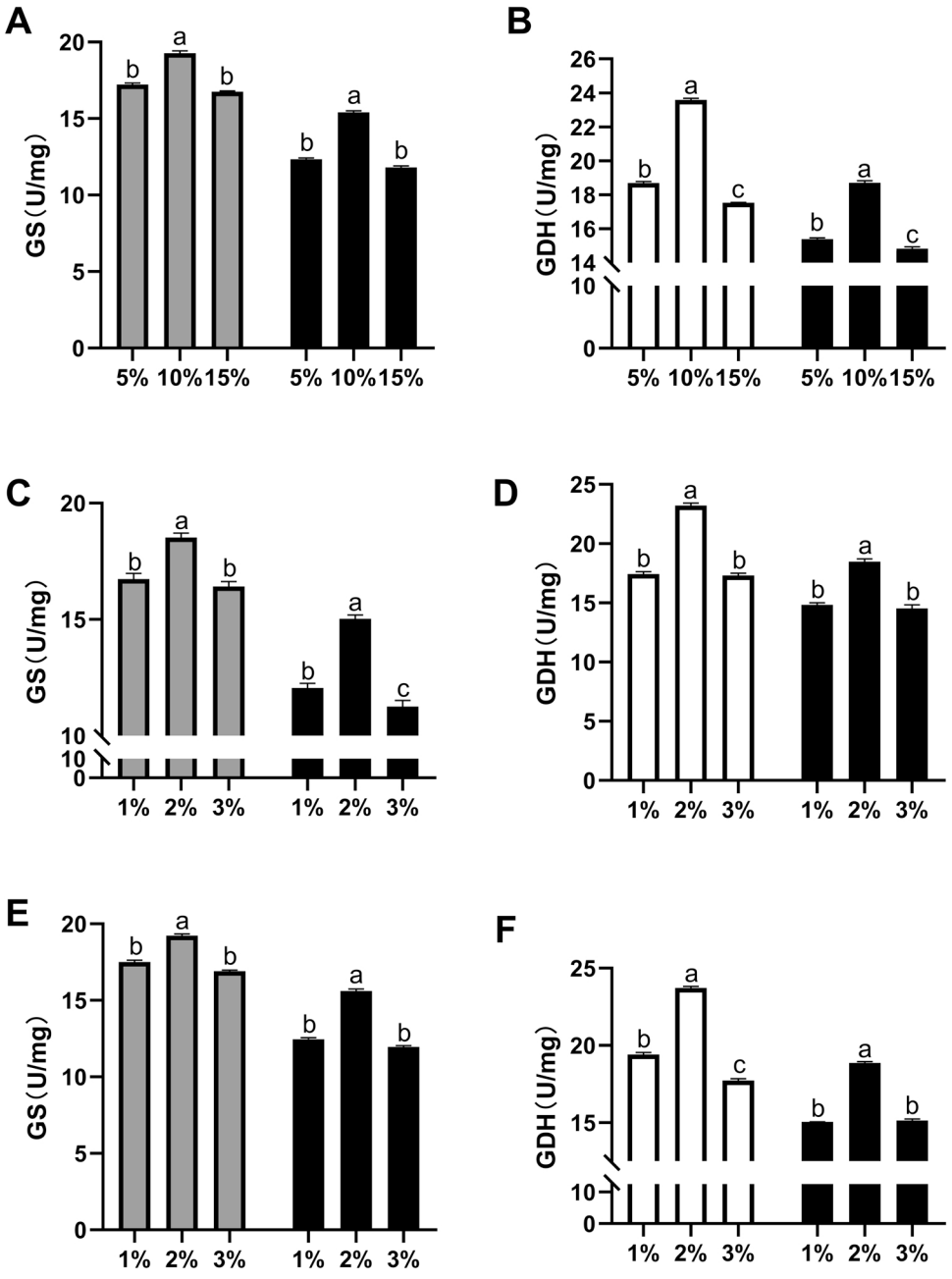

Fig. 3. Enzyme activity of recombinant strains under different induction conditions. Grey represents the *gln*A recombinant strain, white represents the *gdh*A recombinant strain, and black represents the *gdh*A-*gln*A recombinant strain. A, B Enzyme activity of the target protein under culture volumes of 5%, 10%, and 15%; C, D Enzyme activity of the target protein under inoculum sizes of 1%, 2%, and 3%; E, F Enzyme activity of the target protein under methanol concentrations of 1%, 2%, and 3%. Error bars indicate standard error (n = 3). Different letters above the bars indicate statistically significant differences between the samples (ANOVA followed by Duncan's test, $P < 0.05$).

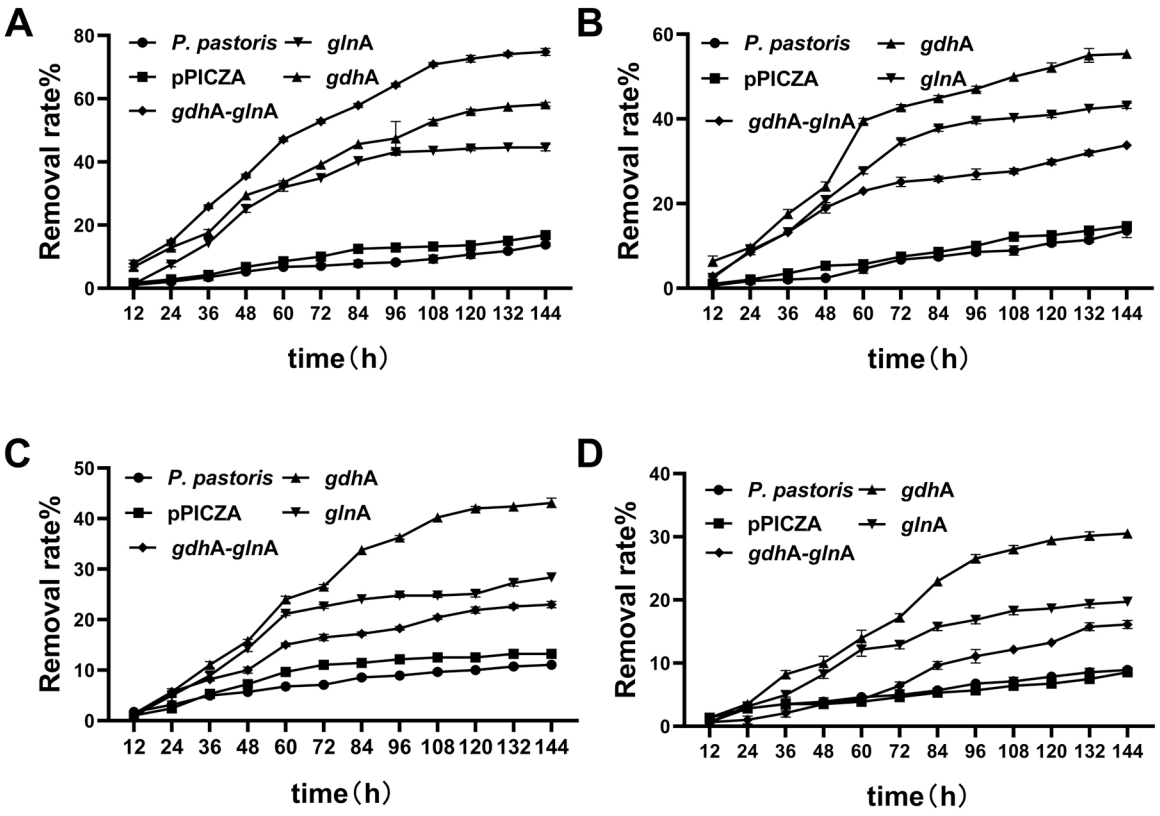

Fig. 4. The removal rate of ammonium nitrogen of each recombinant strain under different initial ammonium nitrogen concentrations after induced expression. A The initial ammonium nitrogen concentration was 0.1 mg/mL. B The initial ammonium nitrogen concentration was 0.3 mg/mL. C The initial ammonium nitrogen concentration was 0.6 mg/mL. D The initial ammonium nitrogen concentration was 1.2 mg/mL. Error bars indicate standard error (n = 3).

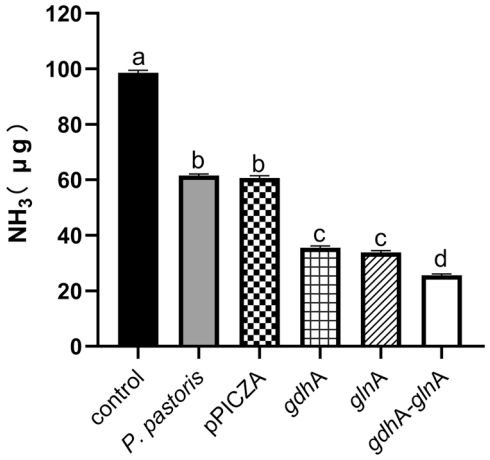

Fig. 5. Effect of recombinant *P. pastoris* strains on the *in vitro* production of NH_3. Error bars indicate standard error (n = 6). Different letters above the bars indicate statistically significant differences between the samples (ANOVA followed by Duncan's test, $P < 0.05$).

to yield two molecules of glutamate (Pengpeng and Tan, 2013). Among them, the *gdh*A-*gln*A recombinant strain can express GDH and GS at the same time, so the emission reduction efficiency is higher than that of single-gene recombinant strains. However, in the medium with a higher ammonium nitrogen concentration, the ammonia-degrading abilities of the double-gene recombinant strain was lower than that of the single-gene recombinant strains. The possible reason is that the activity of the *gdh*A-*gln*A recombinant strain is inhibited by the ammonium nitrogen concentration to varying degrees (Ding et al., 2014). These results indicate that the effect of reducing NH_3 emissions can be achieved by regulating the expression of the *gdh*A and *gln*A genes.

The addition of *P. pastoris* recombinant strains significantly reduced the pH of cecal fermentation broth ($P < 0.05$). Many studies have shown that pH is one of the most important factors affecting NH_3 emissions (Emerson et al., 1975). The reason may be that the GDH and GS in the recombinant strains convert the ammonium nitrogen in the fermentation broth into proteins needed for its own growth, thereby lowering the pH. A lower pH could reduce the transformation of ammonium nitrogen to NH_3 (Emerson et al., 1975). The nitrate nitrogen content in the recombinant strain groups were significantly higher than that observed in the control group. The increase in the nitrate nitrogen content meant that more ammonium nitrogen that could be converted to NH_3 was transformed into more stable nitrate nitrogen, reducing the production of NH_3.

The results of this study also show that the addition of recombinant strains can also reduce the content of uric acid and urea. Microorganisms produce uric acid through nitrogen metabolism. Uric acid is decomposed to produce urea under the action of uricase, and urea is decomposed into NH_3 under the action of urease (Davila et al., 2013). In addition, the addition of recombinant strains can also significantly reduce urease activity involved in the nitrogen-to-ammonia pathway. The reason may be that recombinant strains promote their own proliferation through ammonia assimilation, thereby inhibiting the growth of ammonia-producing bacteria, such as *Klebsiella* and *Proteus* with higher urease activity (Zhang and Kim, 2013). Urease is one of the main rate-limiting enzymes for the production of NH_3 in the degradation of uric acid, and a decrease in urease activity inhibits the decomposition of urea to produce NH_3 (Davila et al., 2013).

The problem of odor pollution has become the main factor restricting the development of the livestock industry. The recombinant *P. pastoris* strains constructed in this study can be used as new additives to reduce

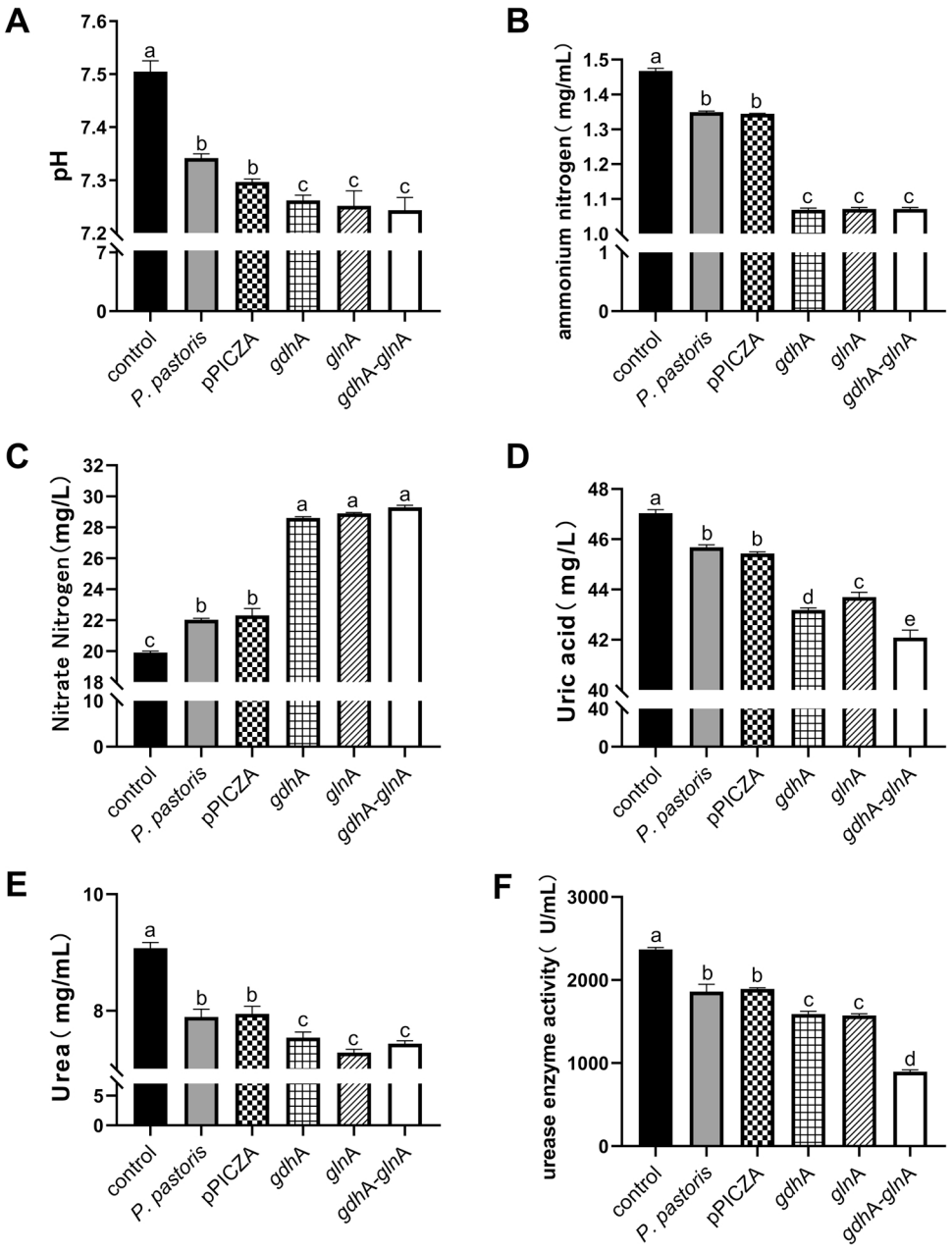

Fig. 6. Effect of recombinant strains on biochemical cecal parameters. A Effect of recombinant strains on the *in vitro* pH. B Effect of recombinant strains on the *in vitro* ammonium nitrogen content. **C** Effect of recombinant strains on the *in vitro* nitrate nitrogen content. D Effect of recombinant strains on the *in vitro* urea acid content. E Effect of recombinant strains on the *in vitro* uric acid content. F Effect of recombinant strains on *in vitro* urease activity. Error bars indicate standard error (n = 6). Different letters above the bars indicate statistically significant differences between the samples (ANOVA followed by Duncan's test, $P < 0.05$).

NH_3 emissions in livestock and poultry. In addition, the ceasing the addition of antibiotics to feed further expands the prospects for the use of these recombinant strains. Our study was based on *in vitro* fermentation, and the actual amount of production and its impact on the performance of livestock and poultry still need to be verified by further experiments.

5. Conclusions

NH_3 emission reduction is an important factor affecting the sustainable development of the livestock industry. Currently, the ammonia emission reduction efficiency of common probiotics is approximately 20–35%. The *gdh*A, *gln*A, and *gdh*A-*gln*A recombinant strains constructed in this study can effectively reduce NH_3 emissions in laying hens, with emission reduction efficiencies as high as 63.95%, 65.68% and 74.04%, respectively. Recombinant strains can degrade ammonia-nitrogen through ammonia assimilation, and reduce NH_3 emissions by reducing the pH, uric acid and urea content of the intestinal tract of livestock and poultry, and urease activity. These results indicate that these recombinant strains have the potential to reduce NH_3 emissions, and are of great significance to reducing the odor pollution caused by the livestock industry.

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CRediT authorship contribution statement

Kunxian Feng: Conceptualization, Data curation, Formal analysis, Methodology, Writing – original draft, Writing – review & editing. **Wei**

Wang: Conceptualization, Investigation, Methodology, Formal analysis, and Data curation. **Jinsheng Rong:** Methodology and Data curation. **Juanboo Liang:** Conceptualization and Supervision. **Jiandui Mi:** Conceptualization and Supervision. **Yinbao Wu:** Conceptualization and Supervision. **Yan Wang:** Conceptualization, Investigation, Writing – review & editing, Resources, Supervision and funding.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.ecoenv.2022.113376.

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