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Highly efficient reduction of ammonia emissions from livestock waste by the synergy of novel manure acidification and inhibition of ureolytic bacteria

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ABSTRACT

The global livestock system is one of the largest sources of ammonia emissions and there is an urgent need for ammonia mitigation. Here, we designed and constructed a novel strategy to abate ammonia emissions via livestock manure acidification based on a synthetic lactic acid bacteria community (LAB SynCom). The LAB SynCom possessed a wide carbon source spectrum and pH profile, high adaptability to the manure environment, and a high capability of generating lactic acid. The mitigation strategy was optimized based on the test and performance by adjusting the LAB SynCom inoculation ratio and the adding frequency of carbon source, which contributed to a total ammonia reduction efficiency of 95.5 %. Furthermore, 16S rDNA amplicon sequencing analysis revealed that the LAB SynCom treatment reshaped the manure microbial community structure. Importantly, 22 manure ureolytic microbial genera and urea hydrolysis were notably inhibited by the LAB SynCom treatment during the treatment process. These findings provide new insight into manure acidification that the conversion from ammonia to ammonium ions and the inhibition of ureolytic bacteria exerted a synergistic effect on ammonia mitigation. This work systematically developed a novel strategy to mitigate ammonia emissions from livestock waste, which is a crucial step forward from traditional manure acidification to novel and environmental-friendly acidification.

1. Introduction

Ammonia emissions are a significant culprit for $PM_{2.5}$ (Li et al., 2016), odor nuisance (Karageorgos et al., 2010), eutrophication (Leinonen et al., 2012), soil acidification (Breemen et al., 1982), biodiversity loss (Pinho et al., 2011), and a hazard to animal and human health (Davidson et al., 2018; Michiels et al., 2015). The global agricultural system is the largest source of ammonia emissions (Behera et al., 2013). The livestock production system contributes to 22–32 Tg N per year, accounting for 50 % of the agricultural emissions (Liu et al., 2022). Particularly, 43 %-70 % of ammonia emissions are from livestock manure (Paulot et al., 2014; Zhang et al., 2017). Therefore, mitigating ammonia emissions from livestock manure has become a priority (Zhang et al., 2020b).

In the livestock manure management chain, urea is quickly hydrolyzed into ammonia and carbon dioxide via urease secreted by fecal ureolytic bacteria after urine is mixed with feces (Sigurdarson et al., 2018). Based on this formation pathway, various ammonia mitigation techniques have been developed mainly including physical, chemical, and biological strategies or a combination of these methods (Emmerling et al., 2020; Hou et al., 2015; Sigurdarson et al., 2018). Currently, covers (physical), urease inhibitors (chemical), and manure acidification (chemical) could reach a high reduction efficiency of over 90 % (Dalby et al., 2020; Sigurdarson et al., 2018). However, covers such as straw

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lead to increased nitrous oxide emissions (Hou et al., 2015). The potential toxic effects of urease inhibitors on soil microbiota and degradation by fecal microbiota (Dalby et al., 2020) are limitations to the wide application on a large and global scale.

In farm-scale application, manure acidification is a recommended technique in some countries of the European Union (Hou et al., 2017; Kai et al., 2008) and China (Guo et al., 2020; Zhang et al., 2019) to reduce ammonia emissions. In past decades, traditional manure acidification is conducted using sulfuric acid which converts ammonia into nonvolatile ammonium ions in manure storage (Fangueiro et al., 2015). However, there are some limitations and criticisms of the application of sulfuric acid, for example, high sulfur input to soils, corrosiveness, and the formation of foam, hydrogen sulfide, and aerosol particles, which is harmful to animals, farmers, and environmental health (Fangueiro et al., 2015; Lehtipalo et al., 2018; Regueiro et al., 2016). Therefore, weak acids such as acetic acid, citric acid, and lactic acid have been employed as alternatives (Overmeyer et al., 2021; Regueiro et al., 2016). Of these, lactic acid possesses a higher acidification capability with a target manure pH of 5.5 (Overmeyer et al., 2021). Nonetheless, a large amount of lactic acid is required to achieve the target pH value and ammonia reduction efficiency due to the lower acidifying capacity compared with sulfuric acid, which leads to a higher treatment cost (Overmeyer et al., 2021). Thus, it is vital to develop a cost-effective strategy to acidify manure with sufficient lactic acid.

Lactic acid bacteria (LAB), widely existing in fermented food (Hu et al., 2020), could generate a high content of lactic acid via the homofermentative pathway (Makarova et al., 2006). In recent years, microbiome engineering has become a powerful tool to synthetically construct microbial communities applied in valuable chemical synthesis and lignocellulose biorefinery (Lawson et al., 2019; Qian et al., 2020). To the best of our knowledge, synthetic microbiome provides a novel idea of mitigating ammonia emissions in the livestock sector. However, it is challenging for the added synthetic lactic acid bacteria community (LAB SynCom) to colonize and acidify manure due to toxins such as antibiotics and negative interactions from manure microbiota (Liu et al., 2020; Zhang et al., 2020a). More importantly, the influence of manure acidification with the extrinsic synthetic microbial community on the manure microbial community succession and this influence on ammonia mitigation are unknown.

Here, we proposed a novel manure acidification strategy based on a LAB SynCom for ammonia mitigation using pig manure as a model. First, we demonstrate the construction of a LAB SynCom with a broad carbon source spectrum and pH profile, high adaptability to the manure environment, and high lactic acid production levels. We next test and optimize the mitigation strategy based on the performance to reach an ammonia reduction efficiency of over 90 % during the entire storage. Finally, influences of the LAB SynCom treatment on the manure microbiota and the ureolytic bacterial community are elucidated using 16S rDNA amplicon sequencing.

2. Materials and methods

2.1. Materials

2.1.1. Manure collection

Fresh feces and urine samples from 7 fattening pigs (Yorkshire, 90–100 kg, 6 months of age) were collected in January 2021 from a typical co-op pig farm with>10-year feeding experience according to the feeding standard of a pillar swine company in Chengdu of Sichuan, the largest pork producing province and an ammonia hotspot in China (Bai et al., 2014; Huang et al., 2012; Pan et al., 2018). All urine and feces samples were homogenized, respectively. These samples were stored at 4 °C for analyzing the microbial population and at −15 °C for conducting the headspace treatment and physicochemical parameters analysis. The physicochemical parameters of feces, urine, and manure are listed in Table. 1.

Table 1
Physicochemical parameters of feces, urine, and manure.

Analysis	Unit	Feces	Urine	Manure
Urea	g/L	1.24	6.41	4.85
TAN	g/L	3.85	1.16	2.43
Reducing sugar	g/L	6.25	–	2.97
pH	–	8.02	8.95	8.3
Lactic acid	g/L	–	–	–
Acetic acid	g/L	6.46	0.84	2.09
Propionic acid	g/L	2.91	0.03	0.58
Butyric acid	g/L	1.65	–	0.35

* Manure was made by urine and feces by a ratio of 3:1 (volume: weight).

2.1.2. Sample collection for screening LAB and strain information

A total of 28 samples including fermented foods and starters for screening functional LAB strains were collected from fermented food producers in 7 provinces of China. Strains screened by this study including *Enterococcus durans* HJ1, *Lactobacillus casei* HJ8, *Lactobacillus paracasei* HJ2, and *Lactobacillus plantarum* HJ3 were deposited in the China General Microbiological Culture Collection Center with accession numbers CGMCC 22591, 23308, 22592, and, 22593, respectively.

2.2. Screening functional strains

28 collected samples of fermented food and starters were each inoculated on 10 solid plates containing different carbon sources. The fast-screening method developed in our previous study (Du et al., 2021) was employed to isolate target LAB strains generating high content of lactic acid on solid plates (observable as yellow circles). Next, liquid media on 96-well plates containing 10 different carbon sources were used to further screen the potent acid producers according to the culture pH. The adaptability of isolated strains to a simulated manure environment was tested via manure solid plates with Oxford cups. The carbon source spectrum of 16 strains was evaluated using liquid manure extract with 10 carbon sources on 96-well plates. The Oxford cup test was employed to assess possible negative pairwise interactions between candidates based on the bottom-up approach (Coyte and Rakoff-Nahoum, 2019). The detailed description for screening functional strains is presented in the supplementary material.

2.3. Headspace emission setup and experimental procedures

2.3.1. Headspace emission setup and operation

Livestock storage simulation systems have been developed to monitor the pH, gas production, and physicochemical parameters (Dalby et al., 2020). In this study, an experimental setup (Fig. 1) was designed to imitate the emissions process of swine manure in a storage tank at room temperature based on the method described by Dalby (Dalby et al., 2020) and National Standard Method for Measuring Ammonia from Air and Exhaust Gas (HJ-533–2009). The experimental setup mainly consists of 6 parts (Fig. 1). The air pumps together with air flow meters and 0.22 μm sterile air filters continuously and steadily dose 0.5 L/min sterile room air (Dalby et al., 2020; HJ-533–2009) via impingers containing 100 mL double distilled water. The humidified air is led via 0.6 mm hoses into the headspace of the reactors. After that, the outlet gas flow from the headspace is directed into 80 mL of 0.1 mol/L sulfuric acid solution which traps ammonia emissions generated from reactors and immediately converts ammonia to ammonium ions for determining ammonia emissions using Nessler's reagent spectrophotometry (HJ-533–2009). The sulfuric acid solution was replaced by fresh sulfuric acid every 24 h to ensure a high absorption efficiency. Before the experiment, all impingers, hoses, and reactors were sterilized by a steam cleaner (Gerllo, Berlin, German) for 30 min. Manure samples of reactors were added, mixed, and collected via the sterile 50-mL injector (Ansairui, Shanghai, China) without opening caps, which avoids microbial contamination while adding and collecting samples. Before the

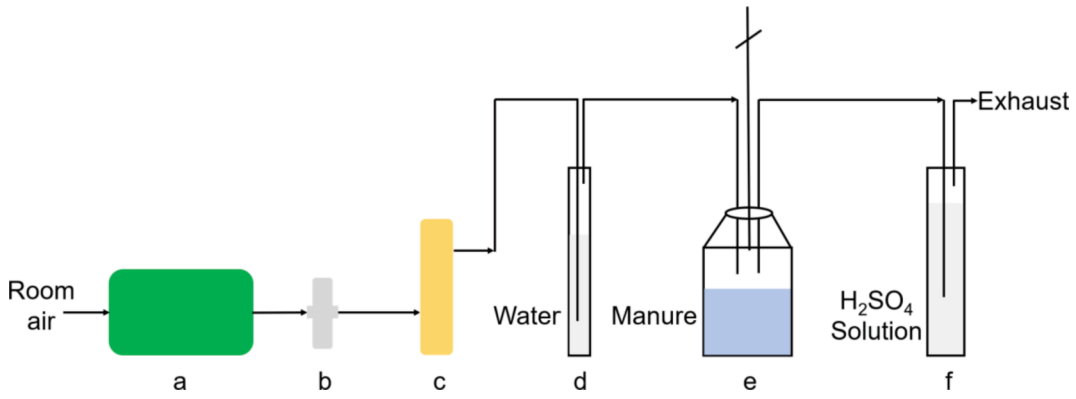

Fig. 1. Experimental headspace setup employed to measure ammonia emissions. (a) Air pumps, (b) air filters, (c) air flow meters, (d) impingers containing double-stilled pure water for humidifying the air, (e) reactors, (f) impingers containing sulfuric acid solution for absorbing ammonia emissions.

treatment, the overall headspace emission setup was run with manure for 12 days to test the running process. The pH of sulfuric acid absorption solution in all impingers was below 2.0, which indicated a high absorption efficiency of ammonia emissions (Fangueiro et al., 2015).

2.3.2. Experimental procedures

Firstly, frozen pig urine and frozen pig feces were thawed in 40 °C water bath based on the reported method (Dalby et al., 2020). Then, the thawed urine and feces by a ratio of 3:1 (volume: weight) (Dalby et al., 2020) were quickly and homogeneously mixed. Immediately after that, 120 mL mixture (manure) was added to each 500-mL reactor.

After that, 12 mL (10 % of manure volume) LAB SynCom was added into reactors and the mixture was homogenized using magnetic stirrers. The treatment commenced when 0.5 L/min ± 0.1 % air stably flowed into reactors. Treatments were conducted at room temperature and were terminated after 12 days, a suggested manure storage duration in pig farms (Dalby et al., 2020). During the treatment process, one mL sulfuric acid absorption solution (0.1 mol/L) was collected every 24 h for determining ammonia emissions, and two mL manure was collected every 24 h for determining physicochemical parameters. All experiments were conducted in three biological replicates.

2.4. Establishment and optimization of the mitigation strategy

2.4.1. The influence of the amount of added lactic acid on manure pH

Lactic acid was used to decrease the initial pH of pig manure from 8.3 to 8.0, 7.5, 7.0, 6.5, 6, 5.5, and 5. The lactic acid was added gradually to pig manure and the mixture was stirred continuously, then the pH of the mixture was measured after each addition using an electrode pH meter (Mettler Toledo, Zurich, Switzerland).

2.4.2. Effects of the LAB SynCom with different inoculation ratios on ammonia reduction efficiency

The inoculation ratio exerts a notable influence on the treatment such as kinetic parameters and microbial activities (Li et al., 2022). In this study, the LAB SynCom together with 21.7 g/L of glucose was added to 120-mL manure containing 3.5×10^8 CFU/mL of bacteria with inoculation ratios of 0.5:1 (1.75×10^8: 3.5×10^8 CFU/mL), 1:1 (3.5×10^8: 3.5×10^8 CFU/mL), and 2:1 (7.0×10^8: 3.5×10^8 CFU/mL), with 10 % of manure volume, respectively. A group only with manure was used as an untreated group. All experiments were conducted in three biological replicates. Treatments were conducted at room temperature and were terminated after 12 days.

2.4.3. Effects of the optimized mitigation strategy on ammonia reduction efficiency

Based on the suggested optimal inoculation ratio and the proposed optimization strategy, a LAB SynCom with an inoculation ratio of 2:1 (population: population) together with 21.7 g/L of glucose was added at the beginning of the treatment. 4.5 g/L of Glucose was added at day 2, 5, and 8 in the treated group. The group without the LAB SynCom but with the same glucose addition frequency was set as the control group, and the group with only manure was set as the untreated group. All experimental conditions and sampling operations were consistent with that of 2.4.2. Two mL manure from the treated and untreated group was collected every 24 h and stored at −20 ℃ for 16S rDNA amplicon sequencing analysis.

2.5. Determination of ammonia emissions and manure physicochemical parameters

Ammonia emissions were measured by Nessler's reagent method for testing the ammonia content of air (HJ-533–2009). The total ammonia nitrogen (TAN) content of manure was measured by Nessler's reagent method for testing that of water (HJ-535–2009). The urea content was determined using the *para*-dimethyl-amino-benzaldehyde (PDAB) reagent method (Roijers and Tas, 1964). The reducing sugar content was measured with the 3, 5-Dinitrosalicylic acid (DNS) reagent method (Miller, 1959). The lactic acid content was determined by the High-Performance Liquid Chromatography (HPLC, Agilent Technologies, CA) method (Ma et al., 2016). Manure pH was measured using an electrode pH meter. The detailed description of methods is presented in the supplementary material.

2.6. Microbial community analysis by 16S rDNA amplicon sequencing

16S rDNA amplicon sequencing was employed to analyze the effect of LAB SynCom treatment on the manure microbial community during the storage. Sample DNA extraction, sequencing data processing, and bioinformatics analysis are described in the supplementary material. The raw sequencing data were deposited into the National Center for Biotechnology Information (NCBI) Sequence Read Archive (SRA) with accession: PRJNA849650.

2.7. Statistical analysis

The difference in lactic acid yield between LAB SynComs was assessed with a Student's *t*-test and differences with $p < 0.05$ were considered statistically significant.

3. Results and discussion

3.1. Strategy design and LAB SynCom construction

3.1.1. Strategy design and performance of functional strains

Manure acidification reduces ammonia emissions by converting

ammonia into nonvolatile ammonium ions (Ndegwa et al., 2008). LAB with homofermentative pathway could generate a high content of lactic acid without the generation of carbon dioxide, acetic acid, and ethanol (Kandler, 1983). Some LAB strains from fermented food could colonize animal feces and are harmless to hosts (Pasolli et al., 2020). Therefore, the strategy with a LAB SynCom generating a high content of lactic acid using the provided carbon sources was designed to achieve acidification efficiently and stably in step 1 (Fig. 2).

A total of 160 potential strains were obtained from around 16,000 colonies on solid media containing 10 carbon sources in step 2 (Fig. 2). Via the further screening process using liquid media, a total of 16 potent strains producing pH of no>5.5 were obtained from the 160 strains. By 16 s rDNA sequencing, 16 strains were assigned to 6 species (Fig. S1) including *Enterococcus durans* (5 strains), *Lactobacillus casei* (1 strain), *Lactobacillus farciminis* (1 strain), *Lactococcus lactis* (2 strains), *Lactobacillus paracasei* (1 strain), *Lactobacillus plantarum* (6 strains).

For application, the adaptability of functional microbes is paramount as manure containing complex abiotic and biotic factors might inhibit added microbes (Liu et al., 2020; Zhang et al., 2020a). Among 16 strains, 9 strains could grow on manure agar plates in step 3. This indicates that they possess high adaptability to manure. Microbes utilizing various carbon sources possess higher competitiveness and adaptability in environments (Egli, 2010). The evaluation of carbon source spectrum (step 4) indicates that six strains could utilize at least four carbon sources. The lactic acid yield for these six strains ranged from 6.4 to 16.2 g/L (Fig. S2). These six strains could adapt to manure with pH levels between 4 and 9. Thus, these six strains were selected as candidates to construct the LAB SynCom.

To construct stable SynComs with desirable functions, negative interactions between candidates should be eliminated as these can affect the community's growth and function (Zhou et al., 2015). In Oxford cup test (step 5), inhibitory zones between the tested strains and background strain coated on plate were not observed for any of the pairs (Fig. S2), so there is no evidence of pairwise negative interactions between 6 candidates on manure extract agar plates. In step 6, 57 LAB SynComs were constructed as mixed cultures of all possible combinations of two, three, four, five, and six candidates.

3.1.2. Constructing the LAB SynCom

Among the 6 mono-cultures, F showed a maximum lactic acid yield of 16.2 g/L. Compared to mono-culture F, the yield of a SynCom was significantly higher with 2 species (SynCom AB, yield 16.9 g/L, $p < 0.05$) and four species (SynCom ABEF, yield 17.2 g/L, $p < 0.05$) and not different with three species (16.6 g/L, $p > 0.05$), five species (16.7 g/L, $p > 0.05$), and six species (16.4 g/L, $p > 0.05$) (Fig. 3). The SynCom with four species including *Enterococcus durans* HJ1 (strain A in Fig. 3), *Lactobacillus casei* HJ8 (strain B), *Lactobacillus paracasei* HJ2 (strain E), and *Lactobacillus plantarum* HJ3 (strain F) possessed the highest lactic acid yield of 17.2 g/L and at a glucose-lactic acid conversion rate of 86 %. This SynCom was therefore selected to mitigate ammonia emissions from manure storage. *Lactobacillus* and *Enterococcus* perform homolactic fermentation (Metselaar et al., 2015; Reddy et al., 2016). Co-culturing homofermentative LAB species could enhance carbohydrate consumption and acidification by nutrient cross-feeding in a chemically synthesized medium (Canon et al., 2021; Ibrahim.,Raman, 2021). These results might explain the synergy of the LAB SynCom on lactic acid production. Moreover, strain A and strain F could generate a higher content of lactic acid with over 6 carbon sources (Fig. S2). In contrast, strain B and strain E better utilized xylan, xylose, and maltose specifically. The wide carbon source spectrum of the LAB SynCom in manure environment provides a

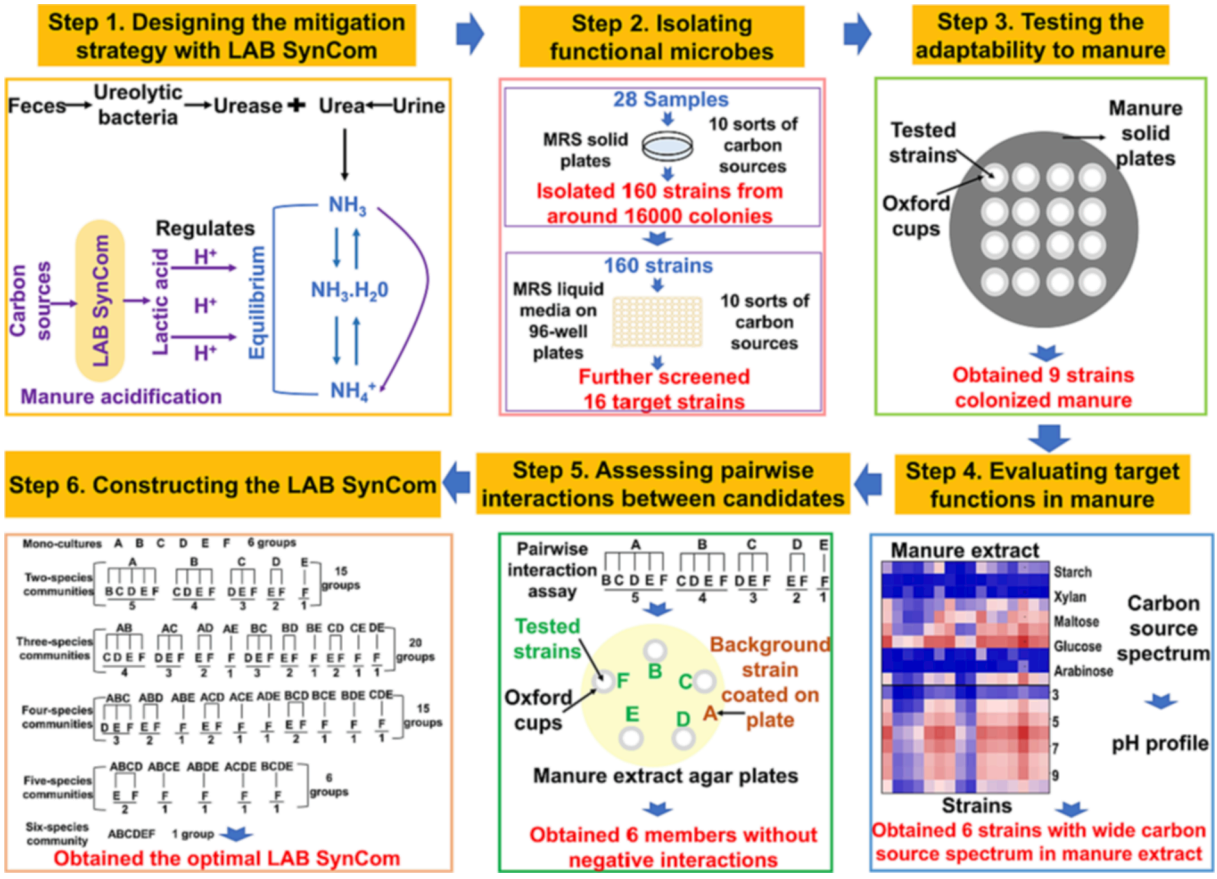

Fig. 2. Strategy design and LAB SynCom construction. Detailed figures and descriptions supporting Fig. 2 are provided in Fig. S1, Fig. S2, and supplementary material.

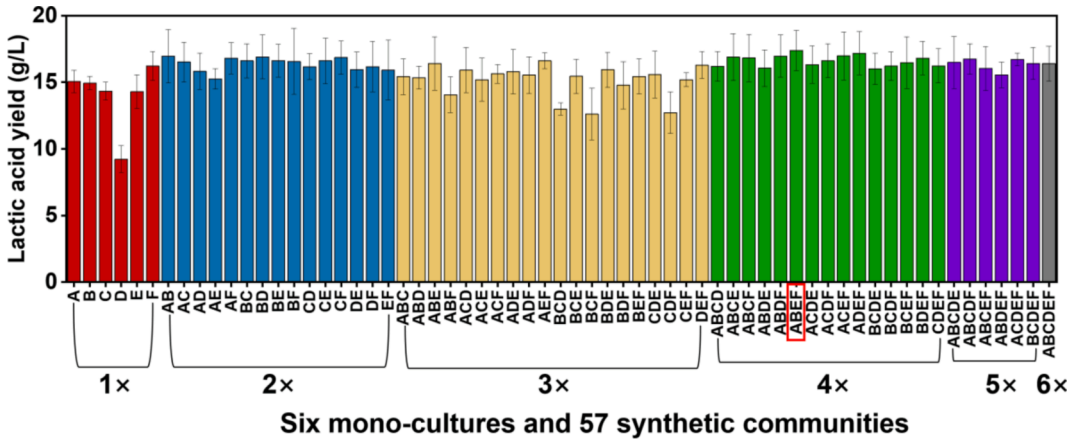

Fig. 3. The lactic acid yield of the 6 mono-cultures (1 ×) and 57 SynComs of two (2 ×), three (3 ×), four (4 ×), five (5 ×), and six strains (6 ×). A represents *Enterococcus durans* HJ1, B represents *Lactobacillus casei* HJ8, C represents *Lactobacillus farciminis* HJ11, D represents *Lactococcus lactis* HJ7, E represents *Lactobacillus paracasei* HJ2, and F represents *Lactobacillus plantarum* HJ3. Data are presented as the mean ± SD of triplicates.

possibility of reducing the mitigation cost by various cheap alternative carbon sources such as sugar beet molasses, pomace, and pretreated straw.

3.2. Establishing and optimizing the mitigation strategy with the LAB SynCom

3.2.1. Establishing the ammonia mitigation strategy with the LAB SynCom

Next, we established the ammonia emission mitigation strategy with the optimal LAB SynCom in real manure. Key process parameters of the manure acidification by the LAB SynCom treatment included target pH, the added amount of carbon source, and the inoculation ratio of the LAB SynCom. The acidification target of 5.5 has been applied to mitigate ammonia emissions in many previous studies (Ndegwa et al., 2008) and it has been proven to be effective with different acidifiers (Kavanagh et al., 2019). Glucose is a common and simple carbon source to generate lactic acid and acidify the environment (Ibrahim.,Raman, 2021; Ratzke and Gore, 2018). Therefore, this study aimed to reduce manure pH from 8.3 ± 0.3 to 5.5 with the LAB SynCom and glucose. Then, we acidified pig manure with lactic acid and determined the needed amount of lactic acid. Based on the linear analysis shown in Fig. 4a, 18.7 g lactic acid should lower the pH of one L manure from 8.3 ± 0.3 to 5.5 (Fig. 4a, blue lines). Based on the glucose-lactic acid conversion rate (86 %) of the optimal LAB SynCom in manure extract, 21.7 g of glucose per L manure was suggested to achieve the acidification target.

The next step is to test the performance of the LAB SynCom under different inoculation ratios. The ammonia emission rate of the untreated group peaked at 61.4 mg/day within two days and then fell to 21.1 mg/day (Fig. 4b) with a total cumulated ammonia emission of 492.1 mg (Fig. 4c). This trend is in accordance with the previous report that urea is quickly broken down to ammonia after mixing feces and urine, and that ammonia is emitted during the entire storage period (Dalby et al., 2020). In treated groups, the total cumulated ammonia emission in groups with inoculation ratios of 1:2, 1:1, and 2:1 was 447.6 mg, 349.1 mg, and 219.8 mg (Fig. 4c), with a corresponding total ammonia reduction efficiency of 9.0 %, 29.0 %, and 55.3 %, respectively. The efficiency increased with the inoculation ratio, a dose-dependent relationship. It is noteworthy that the daily ammonia reduction efficiency achieved over 90 % in the treated group with an inoculation ratio of 2:1 from day 1 to day 3, and continued reducing ammonia emissions until day 9 (Fig. 4d).

To better understand the optimal mitigation strategy, we analyzed the dynamics of pH, lactic acid, reducing sugar, urea, cumulated ammonia emissions, and TAN content of the treated group with the inoculation ratio of 2:1. The treatment process was divided into three stages based on the dynamics of physicochemical parameters (Fig. 4e). In the early stage (day 0 to 3), urea was quickly hydrolyzed from 4.5 g/L to 3.3 g/L (Fig. 4e), and over 90 % of ammonia was converted into ammonium ions achieved by the generated lactic acid from the consumption of reducing sugar (Fig. 4f) which contributed to a quick and notable decrease in manure pH (Fig. 4g). As a result, a considerable formation of TAN was observed (Fig. 4e). In the middle stage (day 3 to 9), ammonia emissions came from the further hydrolysis of urea, down to 1.0 g/L (Fig. 4e). Manure pH value increased during the treatment. The increase in pH is usually attributed to the increase of ammonia (Shin et al., 2019), degradation of organic acids (Regueiro et al., 2016), volatilization of organic acids and carbon dioxide (Clemens et al., 2002), and mineralization of organic nitrogen (Petersen et al., 2012). In this study, pH increased mainly due to the mineralization of organic nitrogen (the TAN increased from 2.50 to 4.2 g/L) in the early stage (Fig. 4e) and the increase of ammonia (Fig. 4e) and degradation of lactic acid (from 10.9 g/L to 1.2 g/L) in the middle stage (Fig. 4f). Thus, adding glucose in the middle stage could be necessary to supply sufficient lactic acid which continued converting ammonia into ammonium ions. In the later stage (day 9 to 12), ammonia emissions came from the conversion of ammonium ions to ammonia with a further increase in manure pH (over 7) and a decrease in TAN content (Fig. 4e). This is in line with the previous study that the increased pH accelerates the formation of ammonia from ammonium ions (Kavanagh et al., 2019).

Based on the functional characteristics and relationship between manure pH and daily ammonia reduction efficiency, we proposed an optimal mitigation strategy. As shown in Fig. 4h, ammonia reduction efficiency reached over 90 % when the pig manure pH was between 5.89 and 6.25. From Fig. 4a and Fig. 4f, the following relations were inferred:

$$\Delta Ca = -k \times \Delta pH \tag{1}$$

$$\Delta Cg = \frac{\Delta Ca}{a} \tag{2}$$

where Ca is the lactic acid concentration (g/L), k is an estimated coefficient (-7.14), Cg is the glucose concentration (g/L), and a is the conversion rate (56.8 %).

Based on these equations, when ΔCa is 2.6 g/L, ΔCg will be 4.5 g/L. Adding this concentration of glucose could maintain the manure pH between 5.89 and 6.25. Manure pH increased back to 6.25 at the third day (Fig. 4h). Therefore, glucose was added every-three days to maintain an ammonia reduction efficiency of over 90 % during the entire treatment process (Fig. 4h).

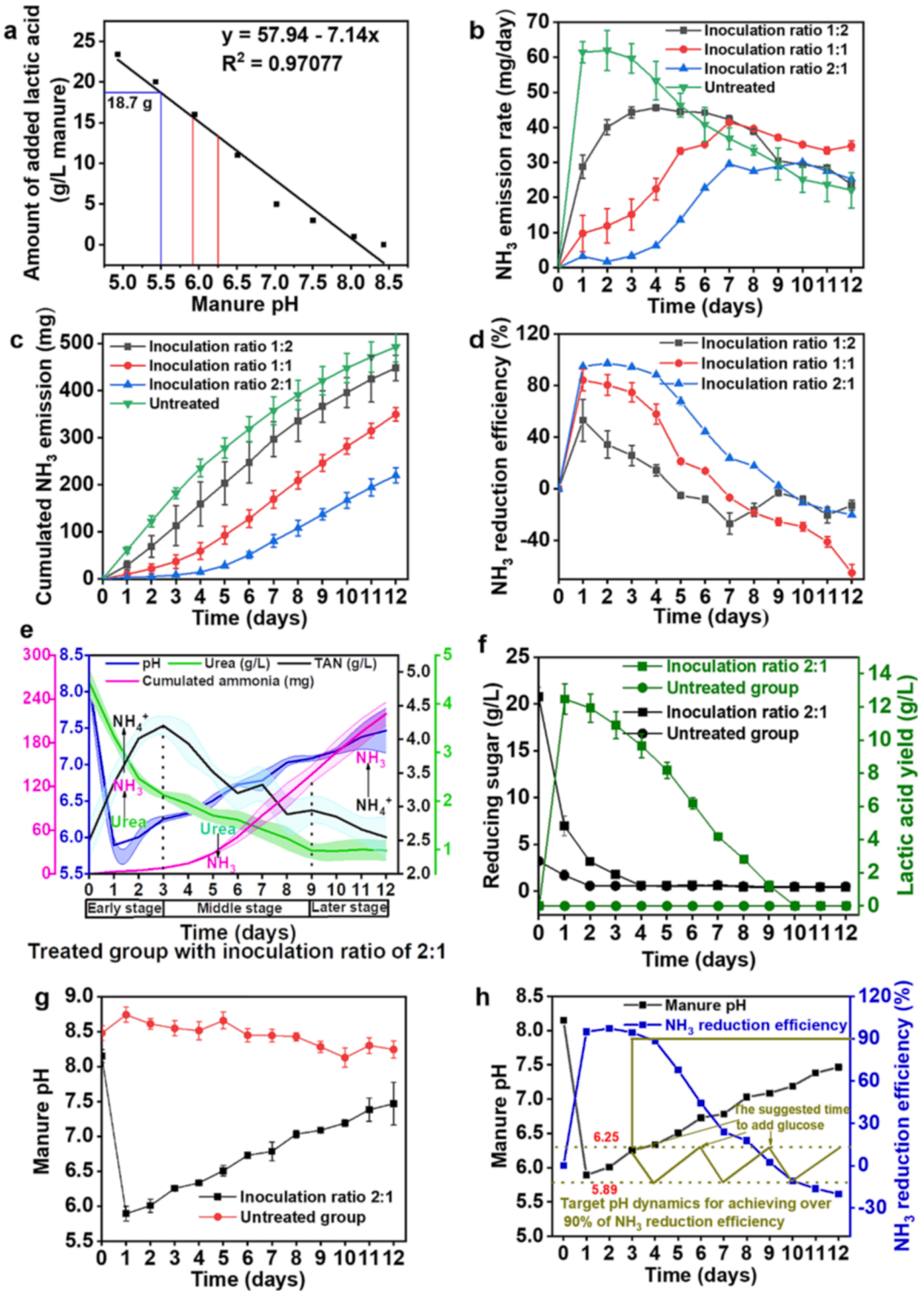

Fig. 4. The effect of the mitigation strategy on ammonia reduction efficiency and manure physicochemical parameters during storage. (a) The influence of the amount of added lactic acid on manure pH, and equation (1) was established, (b) ammonia emission rate, (c) cumulated ammonia emission, (d) ammonia reduction efficiency, (e) integrative analysis of nitrogen-containing compounds of the treated group with the optimal inoculation ratio of 2:1, (f) reducing sugar content and lactic acid yield, and the *in-situ* glucose-lactic acid conversion rate was 56.8 %, (g) manure pH, (h) the proposed optimization strategy. Data are presented as the mean ± SD of triplicates.

3.2.2. *Optimizing the mitigation strategy to enhance the ammonia reduction efficiency*

Next, performance of this optimized mitigation strategy was tested. Based on the optimization strategy suggested in Fig. 4h and the dynamics of manure pH, glucose was added on day 0, day 2, and then every third day with a total of 35.2 g per L manure (Fig. 5a). During the storage period of 12 days, reducing sugar was converted into lactic acid rapidly (within one day) and stably (Fig. 5b and 5c), which maintained the manure pH between 5.58 and 6.05 in the treated group (Fig. 5a). Compared with the untreated group, the maximum ammonia emission

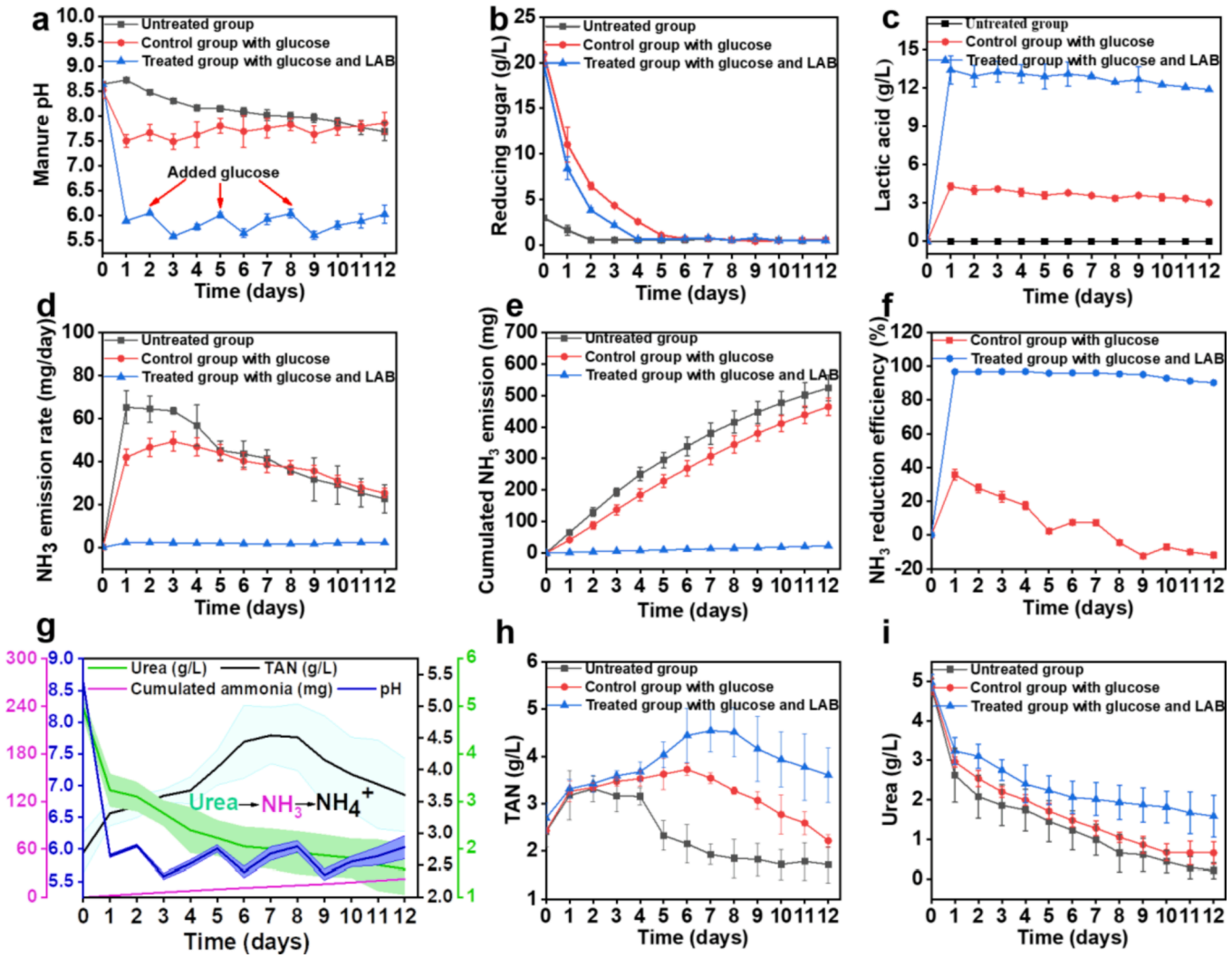

Fig. 5. Ammonia reduction efficiency and manure physicochemical parameters under the optimized mitigation strategy. (a) Manure pH, (b) reducing sugar content, (c) lactic acid content, (d) ammonia emission rate, (e) cumulated ammonia emission, (f) ammonia reduction efficiency, (g) the integrative analysis of nitrogen-containing components of the treated group, (h) TAN content, (i) urea content. Data are presented as the mean ± SD of triplicates.

rate and cumulated ammonia emission did not exceed 2.2 mg/day (Fig. 5d) and 23.2 mg in total (Fig. 5e), respectively. The optimized strategy achieved a total reduction efficiency of 95.5 % and the daily minimum reduction efficiency of over 90 % (Fig. 5f). The maximum daily reduction efficiency and the total reduction efficiency of the control group with glucose only reached 35.8 % and 11.4 %, respectively.

The integrative analysis of nitrogen-containing components showed that urea of the treated group was quickly broken down from 5.0 g/L to 1.6 g/L (Fig. 5g). The cumulated ammonia emissions only slightly increased over time to 23.2 mg, while the TAN content increased rapidly to 4.5 g/L at day 8 and then fell back to 3.6 g/L. At the end of treatment, the TAN content of the treated group was 2.1-fold and 1.6-fold higher than that of the untreated and control group, respectively (Fig. 5h). This result indicates that the LAB SynCom treatment notably reduced the nitrogen loss. At the same time, the urea content of the treated group was 7.5-fold and 2.4-fold higher than that of the untreated and control group, respectively (Fig. 5i), which indicates that urea hydrolysis was inhibited by the LAB SynCom treatment. Dai assessed manure urease activities under pH values between 5 and 9 and reported that urease under lower pH values (5 to 6) shows lower activities (Dai and Karring, 2014). Similarly, Hao reported that manure urease activities would be negatively affected by the low pH (Hao et al., 2019). These results might explain the decrease in urea hydrolysis in this study.

3.3. The influence of the mitigation strategy on pig manure microbial community

3.3.1. Reshaping manure microbial community structure

To gain a comprehensive understanding of the mitigation strategy, we employed 16S rDNA amplicon sequencing to explore the influence of the optimized mitigation strategy on the manure microbial community during storage from day 0 to day 12. There is no significant ($p > 0.05$) difference in Chao1 index (Fig. 6a) between the untreated and treated group at day 0. However, the Chao1 index of the treated group notably decreased and was significantly ($p < 0.001$) lower than that of the untreated group during the storage. This indicates that the SynCom treatment led to a notable decrease in alpha diversity of manure microbiota. In addition, PCoA analysis showed that samples of the untreated and treated group clustered at day 0 while notably separated within one day (Fig. 6b). From day 2 to 12, samples of untreated groups showed a gradual and consistent dispersion as time proceeded while samples of the treated groups clustered (Fig. 6b). The notable difference in beta diversity shows a shift of the microbial community structure between two groups.

Lactobacillus (26.2 %), *Peptoniphilus* (11.2 %), *Corynebacterium* (8.5 %), *Caproiciproducens* (6.7 %), *Peptostreptococcus* (6.5 %), and *Enterococcus* (5.5 %) became dominant genera of the treated group at day 12

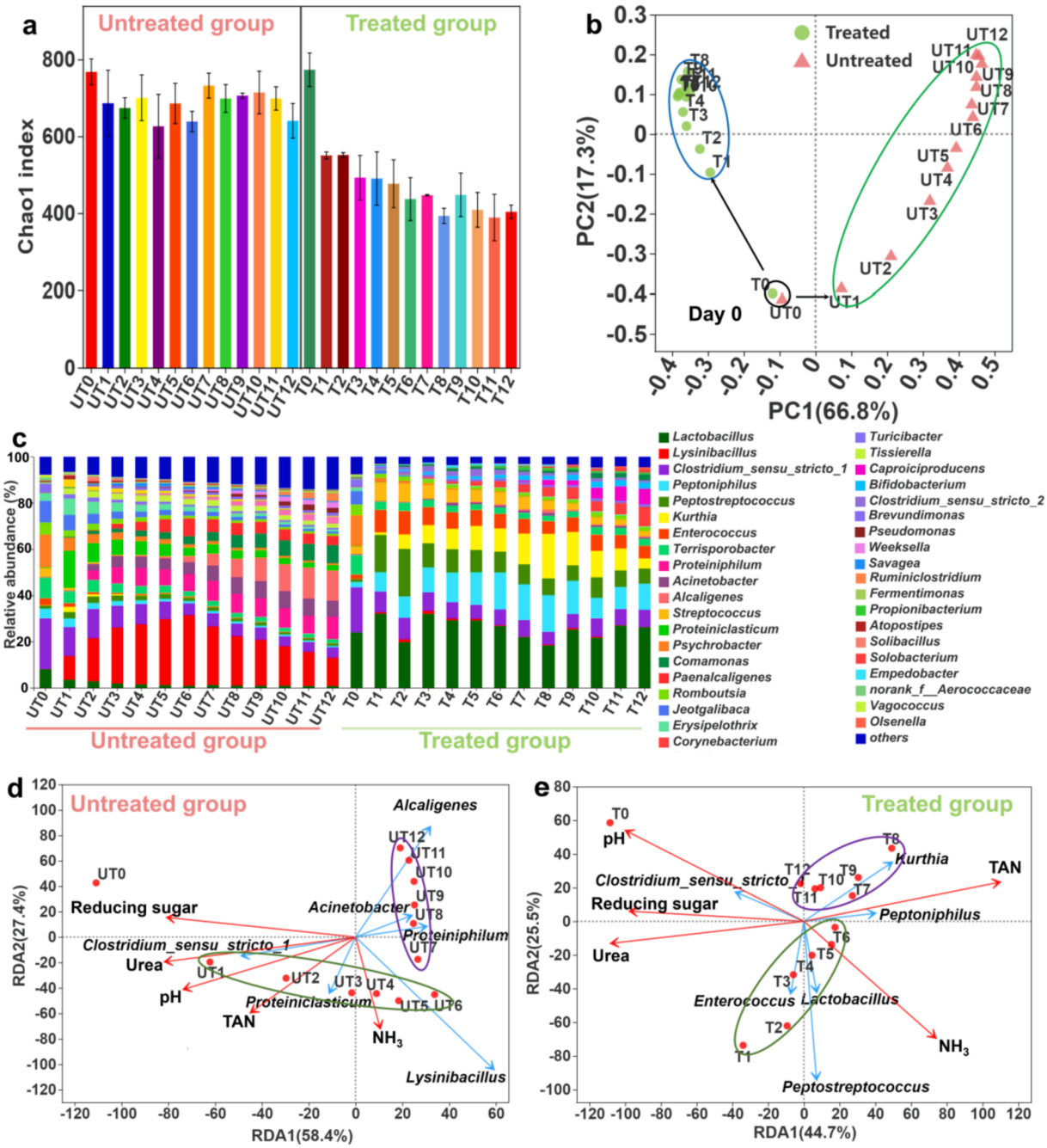

Fig. 6. Effects of the mitigation strategy on the manure microbial community. (a) Alpha diversity by Chao1 index, (b) beta diversity by PCoA based on Bray-Curtis with the significance test based on Adonis, (c) microbial community succession during the storage, RDA of environmental variables and dominant genera of (d) the untreated group and (e) the treated group. Red dots represent samples, red arrows represent environmental variables, and blue arrows represent microbial genera. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

(Fig. 6c). During the treatment process, the increase in relative abundance of *Lactobacillus* and *Enterococcus* was observed after glucose was added at day 0, 2, and 8, respectively (green and orange bars in Fig. 6c). Meanwhile, the total relative abundance of *Lactobacillus* and *Enterococcus* maintained between 23.9 % and 41.9 %. This indicates that these two LAB SynCom genera stably colonized manure. During storage of the untreated manure, the microbial community showed a gradual succession. *Alcaligenes* (13.1 %), *Lysinibacillus* (12.2 %), *Proteiniphilum* (9.8 %), *Comamonas* (7.8 %), *Acinetobacter* (6.8 %), and *Paenalcaligenes* (4.0 %) became the dominant genera at day 12 (Fig. 6c). *Lysinibacillus*, *Alcaligenes*, *Proteiniphilum*, *Acinetobacter*, *Comamonas*, and *Paenalcaligenes*, widely existing in animal guts, feces, or manure (Kim and Lee, 2014; Kunisawa and Kiyono, 2012; Lee et al., 2020; Liu et al., 2020; Wang et al., 2021), accounted for the relative abundance of 53.7 %. On the contrary, the sum of relative abundance of these six genera decreased to only 1.2 % in the treated group. The differences in alpha and beta diversity, and microbial community components between the untreated and treated group indicate that the LAB SynCom treatment reshaped the manure microbial community structure.

Reducing sugar, urea, and ammonium, common carbon and nitrogen sources (Dal Bello et al., 2021; Kuypers et al., 2018; Ricke et al., 1996), together with pH function as important drivers of microbial community succession (Ratzke et al., 2020). The relationship between these environmental variables and microbial communities during the storage was

characterized based on the RDA. Overall, the two axes explained 85.8 % and 70.2 % of the total variance in the untreated and treated group, respectively, suggesting remarkable correlations between microbial communities and these environmental variables. Reducing sugar, urea, TAN, and pH influenced microbial community structure, and ammonia emissions were mainly affected by microbes mainly from day 0 to 6 in both untreated and treated groups (Fig. 6d and e). In the treated group, sufficient glucose provided at day 0 quickly shifted the manure microbial community (Fig. 6b) and decreased manure pH (Fig. 5a) within one day. A small amount of glucose resupplied at days 2, 5, and 8 maintained the high abundance of the LAB SynCom members (Fig. 6c) and low manure pH (Fig. 5a). This result indicates that the mitigation strategy regulated the environmental variables and maintained the stability of the manure microbial community of the treated group, keeping low

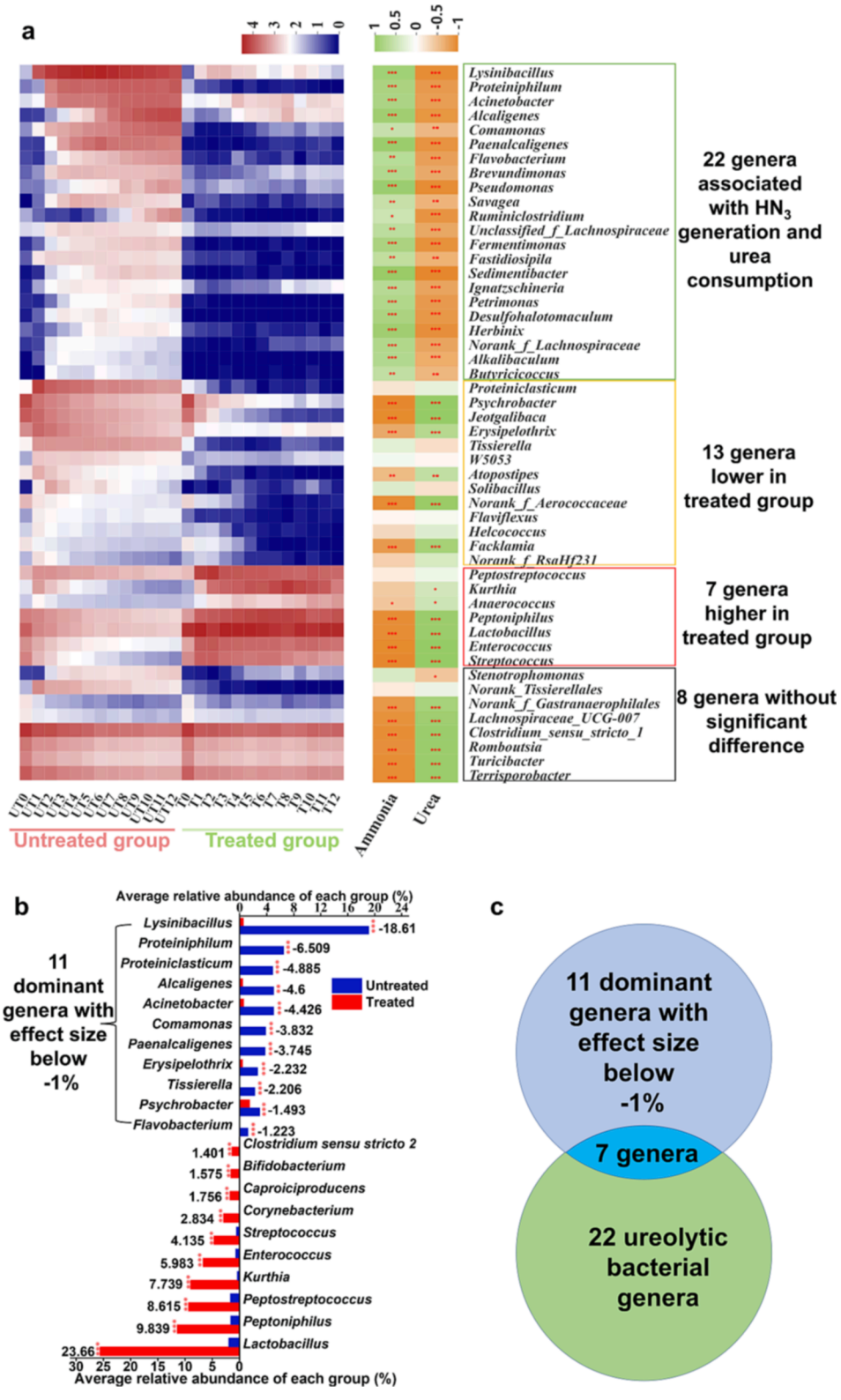

Fig. 7. Effects of the mitigation strategy on manure ureolytic bacterial genera. (a) Heatmaps showing dynamics of the genera abundance (left) and the correlations between each genus and ammonia generation and urea consumption (right) of the untreated group. The top 50 genera of the untreated group are shown. The peak area of each genus shown on the left was normalized using log normalization with $\log_{10}$ (abundance), and the Spearman correlation coefficient is represented by the color, dark green indicates positive correlation, and dark orange indicates negative correlation. P values were calculated using Spearman's rank correlation test, $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$; (b) Genera composition of the untreated group and the treated group with an effect size of>1 % or less than −1%; (c) 7 dominant ureolytic genera identified by the Veen diagram between 22 ureolytic bacterial genera and 11 genera with effect size of below −1%. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

ammonia emissions during the entire storage.

3.3.2. Inhibitory effects of the mitigation strategy on manure ureolytic bacterial community

We further explored the influence of the mitigation strategy on the top 50 manure bacterial genera. The abundance of 35 genera was lower and 7 genera were higher in the treated group than in the untreated group during the storage (Fig. 7a). The Spearman correlation analysis showed that 22 out of 50 genera were associated with both ammonia generation ($\rho > 0.5$ and $p < 0.05$) and urea consumption ($\rho <$ -0.5 and $p < 0.05$) in the untreated group (Fig. 7a). It is reported that *Lysinibacillus*, *Proteiniphilum*, *Alcaligenes*, *Acinetobacter*, and *Comamonas* are ureolytic bacteria (Cui et al., 2018; Hsu et al., 2002; Kim.,Lee, 2014; Li et al., 2019). In the treated group, these 22 ureolytic genera were remarkably decreased compared with their abundances in the untreated group (Fig. 7a). The dissimilarity analysis based on Wilcoxon rank-sum analysis showed that the average relative abundance of these 22 ureolytic genera also significantly ($p < 0.05$) lower in the treated group. Among 35 genera significantly lower in the treated group, the effect size of 11 genera including the dominant genera of *Alcaligenes*, *Lysinibacillus*, *Proteiniphilum*, *Comamonas*, *Acinetobacter*, and *Paenalcaligenes* was below −1% (Fig. 7b). The sum of the average relative abundance of these 11 genera accounted for 60.2 % in the untreated group, while this sum only accounted for 4.2 % in the treated group (Fig. 7a). By contrast, *Lactobacillus* and *Enterococcus*, members of the LAB SynCom, constituted 29.6 % in the treated group (Fig. 7b). As shown in the Veen diagram (Fig. 7c), a total of 7 genera including *Lysinibacillus*, *Proteiniphilum*, *Alcaligenes*, *Acinetobacter*, *Comamonas*, *Paenalcaligenes*, and *Flavobacterium* were identified as dominant manure ureolytic bacterial genera (Fig. 7c). The significant ($p < 0.01$) reduction of these 7 genera in the treated group indicates that the dominant manure ureolytic bacterial genera were inhibited by the LAB SynCom treatment. Lactic acid bacteria possess inhibitions to various microbes in fermented food and animal guts (Ozogul and Hamed, 2018; Vieco-Saiz et al., 2019). The inhibitory mechanisms mainly include the competitive exclusion, production of various antimicrobial compounds, and environmental acidification (van Zyl et al., 2020; Vieco-Saiz et al., 2019). In this study, the LAB SynCom possesses a high adaptability to manure, wide carbon source spectrum, and high nutrient-utilization efficiency. These traits enable LAB SynCom to compete against manure microbes for nutrients and niches. Meanwhile, the LAB SynCom might inhibit the manure microbes by rapidly acidifying manure environment. Future studies should reveal specific inhibitory approaches and interaction mechanisms.

3.3.3. Effects of the mitigation strategy on urea hydrolysis and nitrogen metabolism based on the functional prediction

Based on the observed inhibitory effects on ureolytic bacterial genera, PICRUSt2 was employed for functional prediction of urea hydrolysis, urea cycle, and nitrogen metabolism in the untreated and treated group. This indicates that the mitigation strategy might inhibit both pathways of urea hydrolysis via urease (*P*1, Fig. 8a) and via urease carboxylase together with allophanate hydrolase (*P*2). This prediction was in accordance with the lower and slower breakdown of urea in the treated group, compared with the untreated group (Fig. 5i). The functional prediction also indicates that the predicted functional abundance of the urea cycle (M00029), assimilatory nitrogen reduction ($NO_3^- - NO_2^- - NH_3$), dissimilatory nitrate reduction ($NO_3^- - NO_2^- - NH_3$), and denitrification ($NO_3^- - NO_2^- \rightarrow NO \rightarrow N_2O \rightarrow N_2$) of the untreated group were higher than in the treated group (Fig. 8b). This prediction indicates that the metabolic pathway associated with nitrogen metabolism of producing ammonia might be reduced in the treated group.

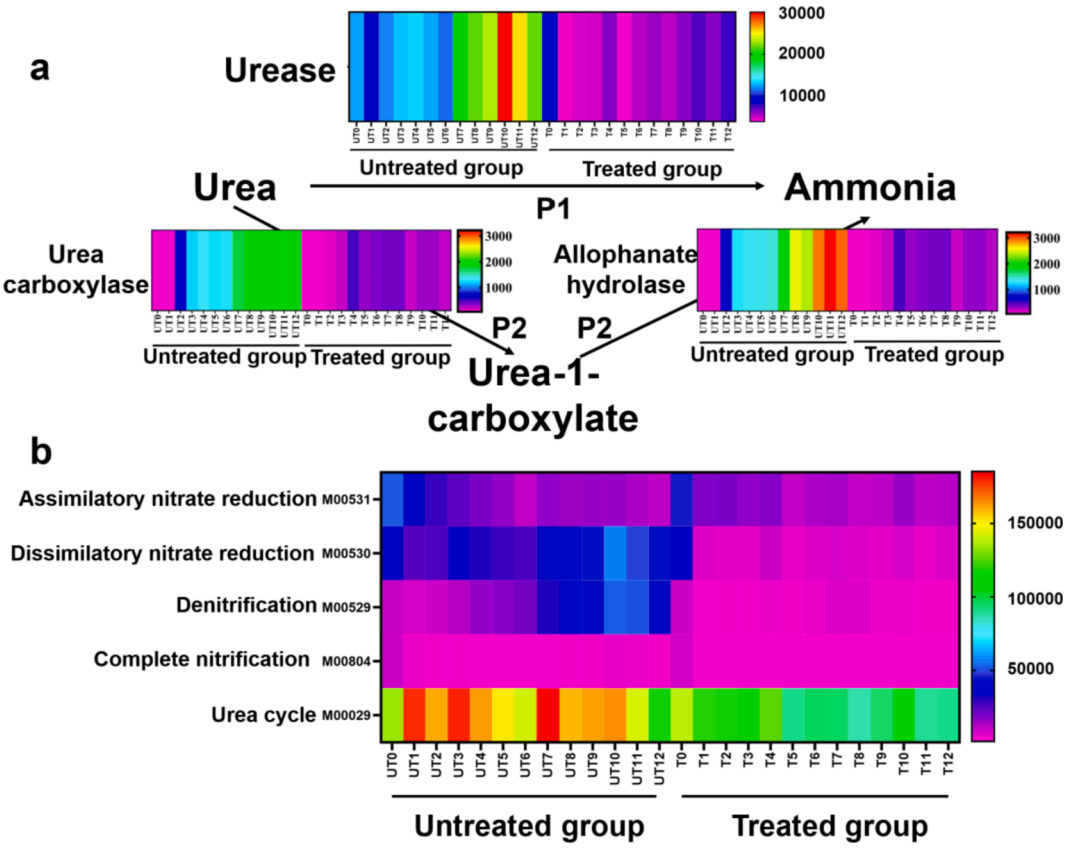

Fig. 8. The potential effect of the mitigation strategy on (a) manure urea hydrolysis and (b) nitrogen metabolism based on the function prediction by PICRUSt2, color bars of the heatmap present the predicted functional abundance.

4. Conclusion

This study developed a novel strategy to mitigate ammonia emissions in the livestock sector via manure acidification based on a LAB SynCom. This mitigation approach achieved a total ammonia reduction efficiency of 95.5 %. As such, it takes a vital step forward in ammonia mitigation from the traditional manure acidification using sulfuric acid to the novel and environmental-friendly acidification. During the treatment process, the LAB SynCom treatment reduced the manure microbial diversity and reshaped the microbial community structure. Importantly, dominant ureolytic bacterial genera were notably inhibited, and the urea hydrolysis was decreased under the mitigation strategy. The conversion from ammonia to nonvolatile ammonium ions and inhibition of ureolytic bacteria exerted a synergy on ammonia mitigation. Our findings provide a novel cognition of ammonia abatement via manure acidification, which exceeded the existing understanding that manure acidification mitigates ammonia emission by converting ammonia into nonvolatile ammonium ions. In future studies, the deeper mitigation mechanism still needs to be uncovered and the mitigation cost of the strategy should be reduced to make it commercially affordable.

CRediT authorship contribution statement

Jun Liu: Conceptualization, Methodology, Investigation, Software, Writing & review. **Xia Li:** Methodology, Investigation, Review. **Yanliang Xu:** Investigation, Performing experiments, Data curation. **Yutian Wu:** Investigation, Performing experiments, Data curation. **Ruili Wang:** Sample collection, Performing experiments. **Xiujuan Zhang:** Sample collection, Performing experiments. **Yaguang Hou:** Sample collection, Performing experiments. **Haoli Qu:** Experimental setup design, Methodology. **Li Wang:** Sample collection, Performing experiments. **Mingxiong He:** Supervision, Review. **Anne Kupczok:** Supervision, Investigation, Review & editing. **Jing He:** Project administration, Funding acquisition, Conceptualization, Writing – review & editing.

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.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.envint.2023.107768.

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