A novel identified *Pseudomonas aeruginosa*, which exhibited nitrate- and nitrite-dependent methane oxidation abilities, could alleviate the disadvantages caused by nitrate supplementation in rumen fluid fermentation

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Summary

After the occurrence of nitrate-dependent anaerobic methane oxidation (AMO) in rumen fluid culture was proved, the organisms that perform the denitrifying anaerobic methane oxidizing (DAMO) process in the rumen of dairy goat were investigated by establishing two enrichment culture systems, which were supplied with methane as the sole carbon source and NaNO₃ or NaNO₂ as the electron acceptor. Several Operational Taxonomic Units (OTU) belonging to Proteobacteria became dominant in the two enrichment systems. The identified *Pseudomonas aeruginosa*, which was isolated from the NaNO₂ enrichment system, could individually perform a whole denitrifying anaerobic methane oxidizing process. Further in vitro rumen fermentation showed that supplementation with the isolated *P. aeruginosa* could reduce methane emissions, alleviate the nitrite accumulation and prevent the decrease in propionic acid product caused by nitrate supplementation.

Introduction

Methane from livestock, especially from ruminants, accounts for 38% of the total emitted greenhouse gases (McGinn et al., 2004), which leads to a loss of 2–12% of the diet energy (Johnson and Johnson, 1995). Some approaches could reduce ruminal methane emission and feed energy loss by inhibiting the synthesis of methane (Gerber et al., 2013; Hristov et al. 2013; Beauchemin et al., 2020). Of these, nitrate supplementation has been shown as the most effective way (Lee and Beauchemin, 2014; Beauchemin et al., 2020). An accepted biochemical mechanism of the effect of nitrate supplementation on reducing ruminal methane emission is that the reduction of nitrite to NH₄⁺ consumes hydrogen while nitrite acts as an intermediate of nitrate metabolism and hence decreases the available hydrogen for methane synthesis in rumen (Nolan et al., 2010). However, because of the richness of nitrate-reducing bacteria in rumen, which leads to the fast conversion of nitrate to nitrite (Lee and Beauchemin, 2014; Beauchemin et al., 2020), the inclusion of nitrate results in nitrite accumulation in rumen and further causes methemoglobinemia when the nitrite is absorbed into the blood. Meanwhile, excessive hydrogen consumption due to the reduction of nitrate and nitrite to NH₄⁺ lead to inhibition of ruminal propionic acid synthesis (Raphélis-Soissan et al., 2017); therefore, further reduces the utilization efficiency of feed energy.

Previous research has found that the ruminal nitrogen increased significantly following dietary nitrate supplementation (Guo et al., 2009), and it indicated that other pathway is also involved in ruminal nitrate metabolism. The DAMO process, which has been widely detected in different environments where nitrate is available (Ettwig et al., 2009; Shen et al., 2014; Wang et al., 2017) and was accelerated by the addition of nitrate (Hatamoto et al., 2017), uses NO₃⁻/NO₂⁻ as the electron acceptor to oxidize CH₄. In this process, both NO₃⁻ and NO₂⁻ can be reduced to N₂. Valdés et al. (1996) and Kajikawa et al. (2010) have reported that AMO process took place in rumen fluid, but both of them did not investigate the electron acceptor for the detected AMO in rumen.

Based on the research evidences above, we proposed that DAMO organisms, including DAMO bacteria (*M. oxyfera*), DAMO archaea (anaerobic methanotrophic-2d, ANME-2d) and anamox bacteria, might inhabit the rumen, which is contacted with the external environment through food and water intake of ruminants. To verify...
our hypothesis, two in vitro enrichment culture systems were established by inoculating the rumen fluid of Saanen dairy goat and meanwhile using methane as the sole carbon source and NaNO₃ or NaNO₂ as the electron acceptor. Furthermore, the effect of the dominant strain in the enrichment culture on reducing the nitrite accumulation induced by nitrate supplementation was detected in in vitro rumen fermentation. Our study might discover another biochemical mechanism of the effect of nitrate supplementation on reducing ruminal methane emission is its stimulative role in the promotion of methane oxidation. Meanwhile, research on the ruminal microorganisms which take part in the DAMO process may provide novel approaches in reducing the accumulation of nitrite induced by nitrate supplementation.

**Results and discussion**

**Anaerobic methane oxidation in the rumen fluid culture**

Nitrate supplementation can effectively decrease ruminal methane emission (Oliéhoek et al., 2016; Granja-Salcedo et al., 2019). The accepted mechanism of this effect is that nitrate competes with CO₂ for hydrogen. However, evidences indicated that AMO exists in all anaerobic environments with plenty of methane, and the category of electron acceptors available determines which kinds of AMO exactly exist in a certain environment (Ettwig et al., 2009; Haroon et al., 2013; Oni and Friedrich, 2017; Valenzuela et al., 2017). Research of Valdés et al. (1996) and Kajikawa et al. (2010) proved that AMO also took place in rumen fluid, yet the electron acceptor for the detected ruminal AMO was not investigated. Giving that nitrate, a common electron receptor in anoxic environment, is available in animal gastrointestinal tract (Kuyper et al. 2018), it is possible that nitrate-dependent AMO also exists in the rumen even without supplemented dietary nitrate and contributes to the nitrate supplementation-induced drop in ruminal methane emission. Based on the previous researches, we hypothesized that nitrate supplementation could promote methane oxidation in rumen. To confirm this hypothesis, the first isotopic tracer experiment was performed. The results showed that the δ¹³C-CO₂ value in the nitrate group was significantly higher than that in the positive control group (Fig. 1A, P < 0.01), which indicated that nitrate promoted the anaerobic methane oxidation in the in vitro rumen fluid.

Proteobacteria became the predominant phylum after long-term enrichment cultivation

As supposed, nitrate promoted AMO in the in vitro rumen fluid. Therefore, more methane should be utilized by DAMO archaea or DAMO bacteria when nitrate is supplemented. However, the expected nitrate-dependent methane oxidation archaea ANME-2d was not detected in the rumen fluid (Liu, 2017). It is possible that its abundance is too low to be detected or some unknown bacteria perform nitrate-dependent methane oxidation in the rumen. The present results suggested that some unidentified bacteria performing anaerobic methane oxidation coupled to nitrate reduction exist in rumen fluid. The results of 16S rRNA gene high-throughput sequencing indicated the bacterial richness and community diversity sharply decreased after eleven months’ enrichment cultivation (Table S1). There were 781 detectable OTUs in the original rumen fluid, while only 247 OTUs were detected in the enrichment system. The abundance of *Proteobacteria* increased from 1.21% (in rumen fluid) to 82.71% after long-term enrichment (Fig. 2A). In addition, the widespread nitrate-dependent ANME-2d, nitrite-dependent *M. oxyfera* and *anammox* were still not detected after 11 months’ enrichment cultivation. These results suggested that the DAMO organism composition in the rumen is different from that in freshwater and wastewater sludge.

The OTU1 (19.38%), OTU2 (31.30%), OTU3 (9.52%) and OTU9 (15.25%) were the most abundant OTUs in the nitrate-dependent methanotroph enrichment culture system, and these OTUs were assigned to *Proteobacteria*. OTU1 was identified as *Paracoccus solventivorans* and unidentified OTU2 and OTU3 belonged to genus *Silanimonas* and *Phenylobacterium* respectively. The sequence of 16S rDNA fragment of OTU9 had 100% similarity with that of *P. aeruginosa*. Mitsumori et al. (2010) detected the methanotrophs inhabiting the rumen by general PCR with *Proteobacterial* methanotroph-specific primers and found new clones close to *Succinivibrio dextrinosolvens*, *Enterobacter cloacae*, *Nitrosomonas sp* and *Actinobacillus minor*, respectively, but none of these new clones was detected in our enrichment culture system.

The OTU28, OTU59, OTU1288 and OTU727, which belonged to the identified methane utilizing bacterial families, became detectable after enrichment culture (see Table S2), but the relative abundance of OTU28 and OTU59 was very low even after eleven months’ enrichment culture. Their low abundance in the enrichment culture and undetectable in the rumen fluid meant they could not play an important role in the ruminal methane oxidation.

Many *proteobacteria* utilize methane under aerobic condition, and it was also reported that the methane oxidation in gammaproteobacterial methanotroph *Methylocomas denitrificans* sp. nov. strain FG1T (GenBank accession no. CP014476) could couple to nitrate reduction under oxygen limitation (Kits et al., 2015). Although no studies have investigated whether bacteria belonging
to genus *Paracoccus*, *Silanimonas*, *Phenylobacterium* and *Pseudomonas* perform AMO, evidences suggested that all of them can carry out nitrate respiration (Baj, 2000; Bartosik et al., 2003; Schreiber et al. 2007; Yong-Sik and Dong-Hyun, 2012; Huang et al., 2013; Engelbrektson et al., 2014).

The OTU6 was assigned to genus *Methanobacterium* and was the only methanogen whose abundance increased after enrichment (Table S2). Most of the identified anaerobic methanotrophs belong to archaea. ANME-2d, which has been characterized by its methane oxidation coupled to nitrate reduction through reverse methanogenesis (Haroon et al., 2013), is one of these ANMEs. The increased relative abundance indicated that OTU6 might carry out methane oxidation through the same way as ANME-2d when methane and nitrate are available in its anaerobic habitat.

Even nitrite-dependent methanotrophs NC10 bacteria was detected in rumen fluid by PCR with NC10-specific primers (Liu, 2017), but they were not found as commensals in the system setting up for enriching ruminal nitrate-dependent methanotrophs. To further study whether there are other unknown nitrite-dependent methanotrophs in the rumen fluid, we established the second enrichment cultivation system by supplying nitrite as the electron acceptor and methane as the sole carbon resource. The most dominant OTU in the culture after 6 months’ enrichment cultivation was taxonomically assigned to *P. aeruginosa* (Fig 2B). As mentioned above, the dominant OTU9 in the first enrichment system was also assigned to *P. aeruginosa*.

**Anaerobic nitrate oxidizing microorganisms were present in the enrichment cultivation system**

Although theoretically the dominant OTUs in the enrichment culture should has the nitrate-dependent AMO ability, no studies to date have reported these dominant OTUs had that ability. Therefore, conclusion cannot be made about whether AMO was still going on in the enrichment system after long-term enrichment cultivation. Then, the second isotopic tracer experiment was carried out by inoculating the subsample of the enrichment culture. As shown in Fig. 1B, the $\delta^{13}$C-CO$_2$ value in the $^{15}$CH$_4$ system was significantly higher than that in the $^{12}$CH$_4$ system after two week’s culture (Fig. 1B, $P < 0.001$). This result confirmed that methane oxidation still occurred in the enrichment cultivation system. The
disappearance of other ruminal methanogens after eleven months' enrichment suggested methane oxidation in the enrichment system was not primarily performed by methanogens through reverse methanogenesis.

The *P. aeruginosa NWAFUP1* strain could perform nitrate-dependent and nitrite-dependent AMO when methane as the carbon resource

Combining the bacterial community structure of the two enrichment cultivation systems, we considered that strains of *P. aeruginosa* in the rumen fluid could perform nitrate-dependent and nitrite-dependent methane oxidation. To confirm this, strains belonged to *Pseudomonas* were isolated from the enrichment cultivation system and were then identified. Ten strains were picked randomly from the plates of *Pseudomonas* selection medium agar. Sequencing and blasting results showed that the 16S rDNA fragments of the ten strains had 100% similarity with that of *P. aeruginosa*. PCR with specific 16S rDNA primer set and *O*-antigen acetylase gene primer set of *P. aeruginosa* confirmed that all of the isolated strains were assigned to *P. aeruginosa* (Fig 3), which also indicated that the main species assigned to OTU1 in the second enrichment system was *P. aeruginosa* rather than other closely related species of *Pseudomonas*.

The third isotopic tracer experiment showed that the $\delta^{13}$C-CO$_2$ and $^{15}$N-N$_2$ value in the $^{13}$CH$_4$ and $^{15}$N-KNO$_3$ system was significantly higher than those in the $^{15}$CH$_4$ and $^{14}$N-KNO$_3$ system after two weeks' culture when the
isolated and identified \textit{P. aeruginosa} NWAFUP1 strain was inoculated in the two culture systems (\( P < 0.001 \)) (Fig. 1C, D). The nitrate consumption and nitrite production of the \textit{P. aeruginosa} NWAFUP1 strain were presented in Fig. S1A. Freedman \textit{et al.} (2004) reported that \textit{P. aeruginosa} can use methanol, ethanol and acetate, but cannot use methane, formate or dichloromethane under denitrifying or aerobic conditions. However, the present strain of \textit{P. aeruginosa} exhibited the ability of anaerobic methane oxidation because of some unknown reasons. In fact, one species of \textit{Pseudomonas} (\textit{Pseudomonas methanica}) was found to be able to utilize methane as a carbon source under aerobic condition (Ferenci \textit{et al.}, 1975). Further study should be conducted to reveal the anaerobic methane oxidation pathway in the isolated \textit{P. aeruginosa} NWAFUP1 strain.

The detected isotopic N\(_2\) in the system inoculated with the isolated NWAFUP1 strain showed that the strain itself could finish the whole DAMO process. The \( \delta^{13}\)C-CO\(_2\) and \( \delta^{15}\)N-N\(_2\) values in the \( ^{13}\)CH\(_4\) and \( ^{15}\)N-NaNO\(_2\) system were significantly higher than those in the \( ^{12}\)CH\(_4\) and \( ^{14}\)N-NaNO\(_2\) system (Fig. 1E, F), and it means that the isolated strain was able to couple methane oxidation to nitrite reduction. This is the first research that found that species of \textit{Pseudomonas} could perform nitrite-dependent AMO under anaerobic conditions when supplied methane as the sole carbon resource. The nitrite consumption in the culture inoculated with the NWAFUP1 strain was presented in the Fig. S1B. The fact that \textit{P. aeruginosa} was the most dominant species in the second enrichment system suggested that it had much higher efficiency of methane oxidation-coupled nitrite reduction than other bacteria. This probably was the main reason why the ruminal NC10 was undetectable in the two enrichment systems after long-term enrichment.

The fact that the relative abundance of the \textit{P. aeruginosa} was 0.03\% of the total bacteria in the original rumen fluid suggested the number of \textit{P. aeruginosa} in the sampled rumen fluid was considerably high. To make it clear whether the high number of ruminal \textit{P. aeruginosa} was just normal, or because the three fistulated goats were infected by \textit{P. aeruginosa}, we investigated the number of \textit{P. aeruginosa} in the rumen of 12 healthy goats and 10 healthy cows. As shown in Fig. 4, each individual had a large number of \textit{P. aeruginosa} in the rumen fluid. Jin \textit{et al.} (2016) once reported that \textit{Pseudomonas}, including the representative specie \textit{P. aeruginosa}, was abundant as the ureolytic bacteria in the rumen. Interestingly, \textit{P. aeruginosa} was not detected in the duodenum and faeces of the corresponding goats and cows (data not showed). We speculated that some bacteria might disappear after they leave the rumen.

\textit{P. aeruginosa} is a well-known opportunistic pathogen and widely distributed, (Molina-Santiago \textit{et al.}, 2019). However, the role of a certain bacterium depends on the specific environment in which it locates and is related to the symbiotic bacteria of this environment. For instance, it was recently reported that \textit{Pseudomonas} and \textit{Bacillus} coexist in plants as probiotics through their respective regulatory mechanisms (Molina-Santiago \textit{et al.}, 2019), and \textit{E. coli}, although it is an opportunistic pathogen, was...
used as a probiotic to reduce methane production and eliminate nitrite accumulation in rumen (Sar et al., 2005), and it was also used for enhanced hydrogen production through gene recombination (Maeda et al., 2012). The high number of ruminal P. aeruginosa strain in each healthy individual indicated it might play an unknown important role in rumen fluid. In fact, Duncan et al. (1999) once reported that P. aeruginosa attached to ruminal epithelial cell in healthy ruminant and protected ruminal epithelial cell by inhibiting the growth of Escherichia coli O157. Our findings indicated AMO occurs in rumen fluid of goat and probably plays an important role in ruminal net methane emission, and it provided another evidence for supporting our hypothesis that supplemented nitrate decreased ruminal methane emission also by promoting ruminal methane anaerobic methane oxidation.

Effect of P. aeruginosa NWAFUP1 strain on the fermentation characters in the rumen fluid which supplemented without or with nitrate in vitro

Rumen acts as an anaerobic microbial fermentation tank. The diet, which arrived at the rumen through the oral cavity, was decomposed into small molecular substances by various types of enzymes secreted by hosts and microorganisms. CH₄ synthesis can reduce ruminal hydrogen partial pressure, therefore increasing the fermentation efficiency of crude fibre (Ungerfeld, 2015). However, ruminal methane emission leads to a loss of 2–12% feed energy and aggravates greenhouse effect (Hu et al., 2015). Supplemented nitrate not only reduces ruminal methane emissions by competing with CO₂ for available hydrogen, but also provides a nitrogen source for microbial protein synthesis by means of its reduction activity of 10 mM nitrate on reducing methane production significantly decreased the methane emission (P < 0.01). Furthermore, nitrate and P. aeruginosa NWAFUP1 strain had an obvious synergistic effect on decreasing methane emission (P < 0.01), but the decreased value was small probably because of the strong effect of 10 mM nitrate on reducing methane production (P < 0.001).

Another obvious negative effect of nitrate addition is its inhibitory effect on synthesis of ruminal propionate (Raphélis-Soissan et al., 2017), which is the main substrate for gluconeogenesis in ruminant. As supposed, the addition of the NWAFUP1 strain tended to alleviate the inhibitory effect of supplemented nitrate on propionate synthesis (P < 0.1) and significantly prevented the decrease of isobutyrate and isovalerate, which was also caused by the inclusion of nitrate (P < 0.05). The NWAFUP1 strain and nitrate had an obvious synergistic effect on decreasing the concentration of NH₃-N (Table 2). It meant that less hydrogen was used for reducing nitrate and nitrite to NH₃-N when nitrate supplemented together with P. aeruginosa NWAFUP1 strain, thus alleviated the inhibitory effect of supplemented nitrate on propionate synthesis.

Conclusion

The main DAMO organisms in rumen fluid were different with those in other environment, and they were identified and unidentified bacteria belonging to Proteobacterial genera which can perform nitrate respiration. The isolated strain of P. aeruginosa NWAFUP1 strain alone could finish the whole process of DAMO and exhibited nitrate- and nitrite-dependent methane oxidation abilities. Supplementation with P. aeruginosa NWAFUP1 strain significantly decreased the methane emission in the ruminal fermentation system in vitro and alleviated the main disadvantages caused by nitrate supplementation.

Experimental procedures

Enrichment cultivation of ruminal nitrate- and nitrite-dependent anaerobic methanotrophs

The enrichment cultivation system was set up by inoculating 20 ml fresh filtered rumen fluid into a 200 ml-
bottle, which contained 60 ml sterile enrichment medium and 5 mM nitrate or 2 mM nitrite. The fresh filtered rumen fluid was obtained by filtering the newly collected rumen fluid through sterile four-layer gauze under anaerobic condition. The rumen fluid was collected from three healthy dairy goats with permanent fistulas and quickly transported to the laboratory. The enrichment medium (per litre) was prepared according to the menu.

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Table 1. Effects of supplement KNO₃ alone or with P. aeruginosa NWAFUP1 strain to rumen fluid on the degradation rate of DM, NDF and ADF in the feed.

| Products produced (%) | KNO₃(-) | P. aeruginosa (-) | P. aeruginosa (+) | KNO₃(+) | P. aeruginosa (-) | P. aeruginosa (+) | P-value |
|-----------------------|---------|------------------|------------------|---------|------------------|------------------|---------|
| DM                    | 52.42 ± 1.87 | 54.41 ± 1.83 | 47.11 ± 0.94 | 51.05 ± 4.46 | 0.021 | 0.086 | 0.537 |
| NDF                   | 36.58 ± 0.32 | 40.20 ± 3.14 | 27.45 ± 2.27 | 38.07 ± 9.26 | 0.005 | 0.001 | 0.044 |
| ADF                   | 31.50 ± 1.03 | 34.40 ± 3.59 | 24.42 ± 2.33 | 35.29 ± 3.71 | 0.100 | 0.003 | 0.043 |

Note: Values are means ± SD. N, Nitrate; P, P. aeruginosa strain, Same as below.

Table 2. Effects of supplement KNO₃ alone or with P. aeruginosa NWAFUP1 strain to rumen fluid on fermentation characters.

| Products produced | 0 mM | 10 mM | P-value |
|-------------------|------|-------|---------|
|                   | Without | With | Without | With | N | P | N x P |
| pH                | 6.92 ± 0.07 | 6.82 ± 0.08 | 7.10 ± 0.09 | 7.09 ± 0.09 | 0.001 | 0.270 | 0.328 |
| Methane           | 10.55 ± 0.88 | 6.87 ± 0.99 | 0.32 ± 0.05 | 0.00 ± 0.00 | 0.000 | 0.001 | 0.002 |
| TVFA (mM)         | 45.66 ± 1.06 | 45.03 ± 5.27 | 38.04 ± 3.51 | 40.85 ± 3.34 | 0.022 | 0.615 | 0.434 |
| Acetate (mM)      | 30.29 ± 0.81 | 30.48 ± 3.67 | 31.77 ± 2.15 | 30.50 ± 0.87 | 0.572 | 0.681 | 0.583 |
| Propionate (mM)   | 9.59 ± 0.20 | 9.21 ± 1.40 | 5.32 ± 1.20 | 8.13 ± 1.75 | 0.007 | 0.139 | 0.062 |
| Isobutyrate (mM)  | 0.50 ± 0.01 | 0.40 ± 0.16 | 0.10 ± 0.02 | 0.29 ± 0.09 | 0.001 | 0.420 | 0.028 |
| Butyrate (mM)     | 3.47 ± 0.09 | 3.39 ± 0.94 | 0.38 ± 0.08 | 0.89 ± 0.44 | 0.000 | 0.490 | 0.354 |
| Isovalerate (mM)  | 0.85 ± 0.01 | 0.72 ± 0.25 | 0.16 ± 0.03 | 0.41 ± 0.10 | 0.000 | 0.476 | 0.044 |
| Valerate (mM)     | 0.96 ± 0.00 | 0.83 ± 0.22 | 0.30 ± 0.14 | 0.64 ± 0.35 | 0.009 | 0.430 | 0.099 |
| Fermentation efficiency (%) | 73.67 ± 0.05 | 73.30 ± 1.13 | 68.79 ± 0.91 | 71.96 ± 1.45 | 0.001 | 0.046 | 0.017 |
| NH₃-N (mg 100 ml⁻¹) | 16.77 ± 0.49 | 19.40 ± 1.64 | 22.72 ± 1.28 | 20.15 ± 0.83 | 0.003 | 0.503 | 0.009 |
| Total bacterial (log-copies per ml) | 11.88 ± 0.16 | 11.73 ± 0.17 | 12.08 ± 0.23 | 12.04 ± 0.08 | 0.048 | 0.438 | 0.654 |
| P. aeruginosa log-copies per ml | 8.28 ± 0.05 | 9.81 ± 0.32 | 9.05 ± 0.23 | 11.74 ± 1.32 | 0.000 | 0.000 | 0.003 |

Fig. 5. Effects of adding nitrate alone, the Pseudomonas aeruginosa NWAFUP1 strain alone or both of it to rumen fluid on nitrate concentration (A) and nitrite concentration (B). Nitrate effect (P < 0.001), Pseudomonas aeruginosa NWAFUP1 strain effect (P < 0.001) and time effect (P < 0.001). Error bars represent standard deviations.
described by Ettwig et al. (2009). Before incubation, oxygen in the bottles was removed by repeatedly pumping-in nitrogen and vacuum-pumping. Then, methane was continuously injected into the bottle to maintain the gas pressure between 1.0 atm and 1.2 atm. All incubations were carried out at 39°C with shaking at 150 rpm.

During the enrichment cultivation, fresh enrichment solution and CH₄ was added under anaerobic condition every 10 days. Firstly, 30 ml supernatant in the bottle was discarded after settlement for one hour, and then, 30 ml fresh enrichment solution was added. Finally, methane was injected into the bottle after oxygen removed. The enrichment cultivation of the two culture systems lasted for 11 months (supplied with nitrate) and 6 months (supplied with nitrite) respectively. At the end of the enrichment cultivation, 10 ml of enrichment culture was harvested for metagenomic DNA extraction.

Strain isolation and sequencing

The 10 ml of collected enrichment culture was serially diluted with sterile phosphate buffer saline (PBS); then, 30 μl of each diluted bacterial culture was coated onto the Pseudomonas CN selective medium plate. Medium CN contained (g l⁻¹): peptone, 16.0; casein hydrolysate, 10.0; potassium sulphate, 10.0; magnesium chloride, 1.4; cetrimonium bromide, 0.2; agar, 15.0. The pH of the medium was adjusted to 7.1 ± 0.2. Ten colons were randomly picked from the solid selective medium plate with scattered colonies of bacteria after cultured for 20 h. Each picked colon was diluted with sterile PBS before inoculated on one solid selective medium plate, and a single colon was picked from each plate. At last, each of the finally isolated strains was cultured in the nutrient broth liquid medium for extracting the bacterial genomic DNA and preserving the strain.

The 16S rDNA fragment of each isolated strain was amplified by using the Pseudomonas-specific primer set of 16S rDNA (Widmer et al., 1998), and PCR amplification products were detected via 1.5% gel electrophoresis and then purified by TIANgel Midi Puri-fication Kit. The purified PCR products were cloned to the PMD19-T vector (TaKaRa Biotechnology, Dalian, China) and transformed into the competent cells of DH5α. One of the positive clones was cultured, and then, the recombined DNA of the plasmid in the cultured positive clone was extracted using TIANprep MiniPlasmid Kit (Tiangen, Beijing, China). Sequencing of the plasmid DNA was completed by bio-engineering (Shanghai) Co., Ltd. The sequences were blasted for comparing the sequence similarity of the amplified fragments with the 16S rDNA sequence of the predominant OTU that was identified as P. aeruginosa. To confirm the isolated and identified strains belonging to P. aeruginosa, the O-antigen acetylase gene fragment of the isolated strains was also amplified by using P. aeruginosa-specific primer set designed by Choi et al. (2013).

Isotope tracer test in rumen system

The first isotopic tracer experiment was carried out to determine the effect of nitrate on anaerobic oxidation of methane in rumen fluid. The experiment contained three groups, including the negative control, the positive control and nitrate group. Each group had three replicates. The rumen fluid of each replicate was prepared in a 135 ml-bottle supplied with 15 ml filtered rumen fluid, 30 ml of artificial saliva (Krishnamoorthy et al., 1991) and 1.0 g alfalfa hay. The three groups were supplemented with 4 mM NH₄Cl (negative control), 4 mM NH₄Cl (positive control) and 4 mM nitrate (treatment group) respectively. Before incubation, oxygen in the bottle was removed by repeatedly pumping-in nitrogen and vacuum-pumping. Then, 15% ¹³CH₄ (Sigma-Aldrich, St. Louis, MO, USA, 99 atm % ¹³C) and 85% N₂ was pumped in the headspace of bottle of the positive group and the treatment group to maintain gas pressure between 1.0 atm and 1.2 atm. Fifteen per cent of ¹²CH₄ (Sigma-Aldrich, 99 atm % ¹²C) and 85% N₂ was pumped in the negative group. All bottles were incubated at 39°C with shaking at 150 rpm for 24 h. Gas in the headspace of each bottle was collected with an injection syringe and transferred to the vacuum bag for testing the δ¹³C-CO₂ value.

Isotope tracer test in the enrichment culture system

The second isotopic tracer experiment was carried out to verify the existence of anaerobic methane oxidation in the enrichment cultivation system. This experiment contained a control group and an isotope group, each group had three replicates. Each replicate bottle (135 ml) was contained 50 ml of the enrichment medium and 10 ml subsample of enrichment culture. After oxygen removed, 96 ml ¹³CH₄ was injected in the isotope group, and 96 ml ¹²CH₄ was injected in the control group. The bottles were cultured at 39°C at 150 rpm for 30 days. Gas in the headspace of the bottle was collected for testing the δ¹³C-CO₂ value. To prepare the subsample of the enrichment cultivation systems, 20 ml enrichment culture was added in a new bottle that contained 60 ml enrichment solution and then anaerobically incubated for 2 weeks after methane pumped in.

Isotope tracer test in the culture system inoculated with the isolated NWAFUP1 strain

The third isotopic tracer experiment was carried out to check on the existence of nitrate- and nitrite-dependent anaerobic oxidation of methane in the culture system
inoculated with the isolated NWAFUP1 strain (GenBank accession no. MK673864), whose 16S rDNA fragments had 100% sequence similarity with P. aeruginosa. The experiment contained one control group and one tracer group. Each group had three replicates. Each replicate bottle (135 ml) was combined with 55 ml of anaerobic buffer solution supplemented with 4 mM N-KNO₃ (¹⁵N-KNO₃ in the tracer group, and ¹⁴N-KNO₃ in the control group). After oxygen removed by repeatedly injecting 99.99% N₂ and vacuum pumping, 96 ml ¹³CH₄ was injected into the bottles of the tracer group, and 96 ml ¹²CH₄ was injected into the control group. All the bottles were then cultured at 39°C with shaking at 150 rpm for two weeks. Gas in the headspace of the bottles was collected for detecting ¹³C-CO₂ and ¹⁵N-N₂. The concentrations of NO₂ and NO₃ in the liquid were detected every seven days.

The ability of nitrite-dependent anaerobic methane oxidation of the NWAFUP1 strain was also determined by supplying 2 mM N-NaNO₂ (¹⁵N-NaNO₂ in the tracer group, and ¹⁴N-NaNO₂ in the control group) in the anaerobic cultivation system. The remaining of the operation was followed the steps above.

In vitro fermentation
The 2 × 2 Latin square was designed for investigating the effect of the NWAFUP1 strain supplemented alone or with nitrate together on the ruminal fermentation characters and nitrite accumulation. The fresh filtered rumen fluid was prepared according to the method mentioned above. Fifteen milliliters of filtered fluid were inoculated into each of the 48 anaerobic bottles (135 ml ml), which contained 20 ml of artificial saliva (Krishnamoorthy et al., 1991) and 0.5 g alfalfa hay. The bottles were randomly assigned to four groups, including the control group (only supplement 5 ml physiological saline), the P. aeruginosa NWAFUP1 strain group (final concentration of P. aeruginosa NWAFUP1 strain was 5 × 10⁷ CFU ml⁻¹), the KNO₃ group (10 mM), and the P. aeruginosa NWAFUP1 strain (final concentration of P. aeruginosa NWAFUP1 strain was 5 × 10⁷ CFU ml⁻¹) + KNO₃ (10 mM) group. Each group had 12 bottles. All the bottles were balanced to an atmospheric pressure after oxygen removed and then cultured at 39°C with shaking for 24 h. Three bottles of each group were selected at 0, 4, 8, 12 and 24 h to measure nitrate and nitrite concentration.

DNA extraction
The modified Cetyltrimethylammonium Ammonium Bromide (CTAB) method (Liu et al., 2017) was used to extract metagenomic DNA from the mixed original rumen fluid, the enrichment culture system and the fermentation samples. The purity and concentration of the extracted DNA samples were detected by 1% agar gel electrophoresis and a micronucleic acid analyser (NanoDrop 2000, Thermo Fisher Scientific, Inc., Madison, WI, USA) and then stored at −20°C until analysis.

High-throughput sequencing
The 16S rDNA fragment was amplified from metagenomic DNA and sequenced using the Illumina HiSeq2500 high-throughput sequencing system. The oligonucleotide primers for high-throughput sequencing were 341b4-F (5'-CTAYGGRBGCWGAGC-3') and 806-R (5'-GGACTTACNNGGTATCTAA-3') (Lu et al., 2015). Each sample contained at least 40 000 clean oligonucleotides. Quality filtering on raw tags was performed using specific filtering conditions to obtain high-quality clean tags with QIIME software (Tanjana and Salzberg, 2011). The operational taxonomic units (OTUs) were based on ≥ 97% sequence similarity (Li et al., 2017). The a-diversity of the samples (the rarefaction, Chao1 richness, Goods-cover- age, Simpson and Shannon diversity indices) was calculated with QIIME (Caporaso et al., 2010).

Quantitative PCR (qPCR)
The abundance of total bacteria and P. aeruginosa was analysed in a Bio-Rad-iQ5 (Bio-Rad, Laboratories Inc., Hercules, CA, USA). Each PCR reaction system (20 μl) contained 1 μl of the corresponding 10 μM upstream and downstream primers (Table S3), 1 μl of 30 ng DNA, 10 μl SYBR Green I (TAKARA BIO, Inc., Kyoto, Japan) and 7 μl of distilled water. The standard DNA sample and the standard curve of absolute quantitative PCR were prepared by referring to the method described by Liu et al. (2017).

Chemical analysis
The value of ¹³C-CO₂ and ¹⁵N-N₂ was determined by gas chromatography–combustion/thermal conversion–isotope ratio mass spectrometry (GC-C/TCII-IRMS). The international sample VPDB (Vienna Peedee Belemnite) was used as the standard sample (Liu et al., 2017). The concentration of nitrate and nitrite were measured by spectrophotometer, according to Cataldo et al. (1975) and He. (2016).

The pH value of the fermentation samples was measured by a pH meter (PHS-3C, Shanghai Precision Scientific instrument Co., Ltd.). Then, the fermentation samples were filtered and divided into several parts and preserved at −80°C to determine the concentrations of nitrate, nitrite, volatile fatty acids (VFA), NH₃-N, the number of total bacteria and P. aeruginosa. The
concentration of VFA was determined by gas chromatography (Agilent Technologies 7820A GC system, Santa Clara, CA, USA), and the detailed method was described by Li et al. (2014). The fermentation efficiency was calculated as (0.62 acetate + 1.09 propionate + 0.78 butyrate)/(acetate + propionate + butyrate) ×100 (Chalupa, 1977). The concentration of NH₃-N was determined via phenol-sodium hypochlorite colorimetric method described by Chaney and Marbach (1962).

The content of NDF and ADF was analysed by Ankom® A200I fibre analyser (ANKOM Technology, Macedon, NY, USA), according to the filter bag technique mentioned by Van Soest et al. (1991). The degradation rate of NDF and ADF was calculated by the method described by Li et al. (2014).

In addition, the gas produced by fermentation for 24 h was collected with a calibrated glass syringe and its composition was determined using gas chromatography mentioned by Wang et al. (2017) and Yang et al. (2012) (Japan, Shimadzu, GC-2014), while the concentration of CH₄ was calculated from gas production.

Statistical analysis

The δ¹³C-CO₂ mean value in the first isotopic tracer experiment was analysed using the one-way ANOVA of SPSS (Statistical Package for the Social Sciences) 23.0 software (Chicago, IL, USA) to test the significant differences among the groups. The δ¹⁵N-N₂ and δ¹³C-CO₂ mean values in the second and the third isotopic tracer experiments were analysed using the t-test procedure to test the significant differences between the two groups.

The figures were used to analyse the in vitro ruminal fermentation test, the NO₃ and NO₂ concentration by using two-way repeated measures ANOVA. In brief, each time point was the repeated factor, the KNO₃ and the NWAFUP1 strain were the double factors to observe the dynamic changes of NO₃ concentration and NO₂ concentration. Other data, such as CH₄, DMD and VFA, were analysed using the two-way ANOVA of SPSS. All data are expressed as the means with the standard deviation. The difference was considered to be statistically significant at \( P \leq 0.05 \).

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Conflict of interest

The authors have no conflicts of interest to declare regarding this work.

Ethical approval

All applicable international, national and institutional guidelines for the care and use of animals were followed.

Author contributions

Pang J, Liu LH and Xu XR designed the experiment, performed the enrichment culture and two isotopic tracer experiments and detected the diversity of NC10. Pang J and Liu XP isolated and identified the strain of OTU9, performed the third isotopic tracer experiments. Wang Y and Chen B helped Pang J isolate and identify the strain of OTU9. Xu XR helped Pang J and Liu LH design the experiment, analysed the sequencing data and wrote the manuscript. Yao JH and Wu SR helped design the experiment and revised the article.

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