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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 $NaNO_3$ or $NaNO_2$ 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 $NaNO_2$ 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 NH_4^+ 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 NH_4^+ 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 NO_3^-/NO_2^- as the electron receptor to oxidize CH_4. In this process, both NO_3^- and NO_2^- can be reduced to N_2. 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 *anammox* bacteria, might inhabit the rumen, which is contacted with the external environment through food and water intake of ruminants. To verify

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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 $NaNO_3$ or $NaNO_2$ 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 (Olijhoek *et al*., 2016; Granja-Salcedo *et al*., 2019). The accepted mechanism of this effect is that nitrate competes with CO_2 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 (Kuypers *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 δ^{13}C-CO_2 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 *Methylomonas denitrificans sp*. nov. strain FJG1T (GenBank accession no. CP014476) could couple to nitrate reduction under oxygen limitation (Kits *et al*., 2015). Although no studies have investigated whether bacteria belonging

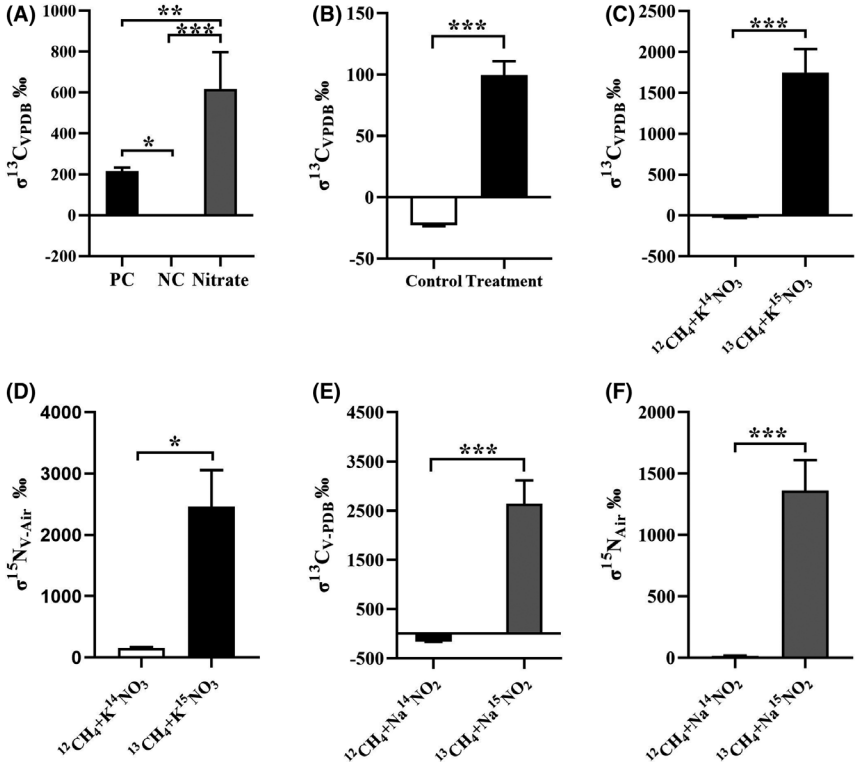

Fig. 1. The $\sigma^{13}C$-CO_2 and $\sigma^{15}N$-N_2 value in three isotope tracer experiments. A. Effect of nitrate on AMO in the rumen fluid culture. B. Effect of nitrate on AMO in the enrichment culture system. C, D. Effect of nitrate on AMO in the NWAFUP1 strain culture.E, F. Effect of nitrite on AMO in the NWAFUP1 strain culture.
Note: (A) NC, negative control; PC, positive control.
***, $P < 0.001$, **, $P < 0.01$; *, $P < 0.05$, same as below.

to genus *Paracocccus*, *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 $^{13}CH_4$ 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

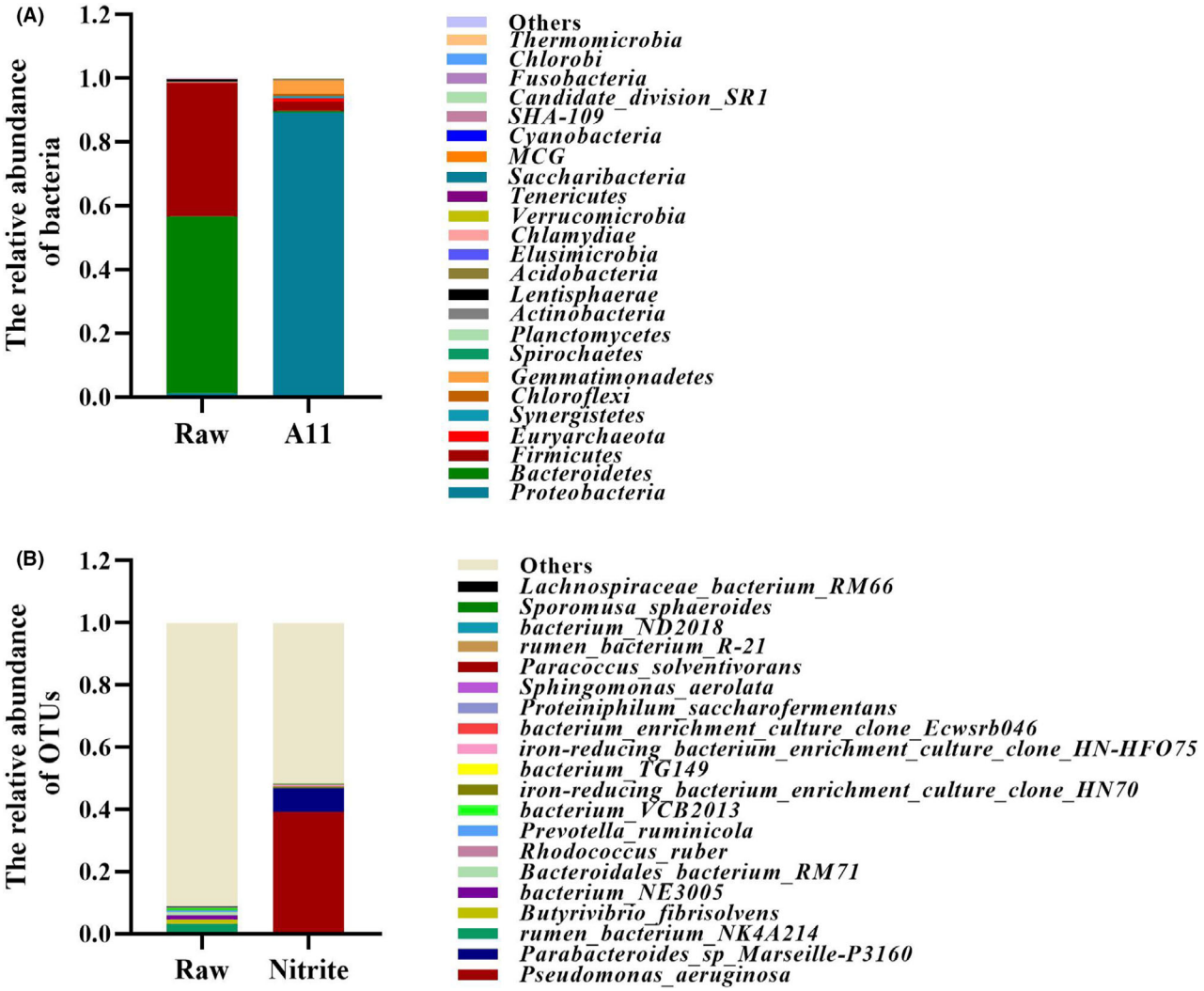

Fig. 2. The relative abundance of the top twenty bacteria in the nitrate- and nitrite- dependent methanotrophs in rumen fluid and enrichment cultivation system.A. Bacterial community structure at the phyla level in the nitrate-dependent methanotrophs enrichment cultivation system after 11 months; (B) Bacterial community structure at the OTUs in the nitrite-dependent methanotrophs enrichment cultivation system after 6 months. Note: The sample collected from the enrichment cultivation system after eleven months was named as A11, and the original rumen fluid was named Raw.

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 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 δ^{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 $^{12}CH_4$ and ^{14}N-KNO_3 system after two weeks' culture when the

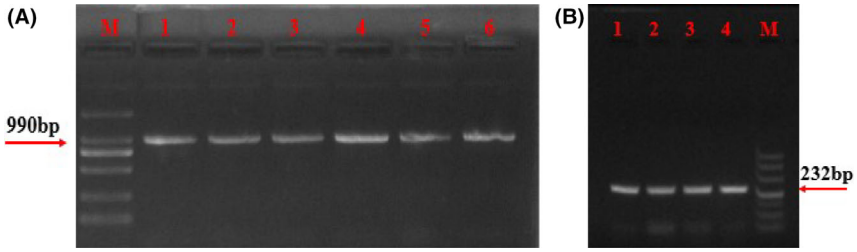

Fig. 3. The target fragments of 16S rDNA and *O-antigen acetylase* gene of the strains isolated from the enrichment cultivation systems.A. PCR products of the picked strains 16S rDNA using *Pseudomonas*-specific primers.B. PCR products of the picked strains using *Pseudomonas aeruginosa O-antigen acetylase* gene-specific primers.

isolated and identified *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 *P. aeruginosa* NWAFUP1 strain were presented in Fig. S1A. Freedman *et al*. (2004) reported that *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 *P. aeruginosa* exhibited the ability of anaerobic methane oxidation because of some unknown reasons. In fact, one species of *Pseudomonas* (*Pseudomonas methanica*) was found to be able to utilize methane as a carbon source under aerobic condition (Ferenci *et al*., 1975). Further study should be conducted to reveal the anaerobic methane oxidation pathway in the isolated *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 $\delta^{13}C\text{-}CO_2$ and $\delta^{15}N\text{-}N_2$ values in the $^{13}CH_4$ and $^{15}N\text{-}NaNO_2$ system were significantly higher than those in the $^{12}CH_4$ and $^{14}N\text{-}NaNO_2$ 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 *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 *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 *P.aeruginosa* was 0.03% of the total bacteria in the original rumen fluid suggested the number of *P. aeruginosa* in the sampled rumen fluid was considerably high. To make it clear whether the high number of ruminal *P. aeruginosa* was just normal, or because the three fistulated goats were infected by *P. aeruginosa*, we investigated the number of *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 *P. aeruginosa* in the rumen fluid. Jin *et al*. (2016) once reported that *Pseudomonas*, including the representative specie *P. aeruginosa*, was abundant as the ureolytic bacteria in the rumen. Interestingly, *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.

P. aeruginosa is a well-known opportunistic pathogen and widely distributed, (Molina-Santiago *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 *Pseudomonas* and *Bacillus* coexist in plants as probiotics through their respective regulatory mechanisms (Molina-Santiago *et al*., 2019), and *E. coli*, although it is an opportunistic pathogen, was

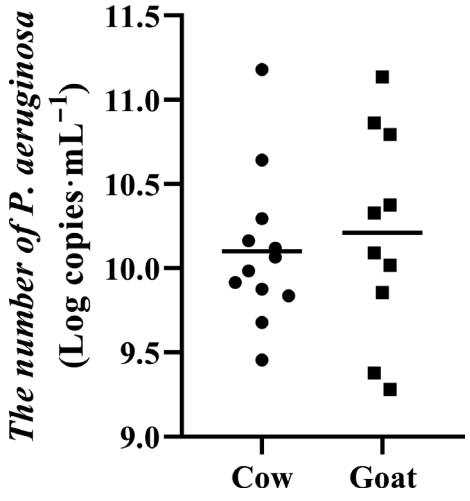

Fig. 4. Number of *Pseudomonas aeruginosa* in rumen fluid of healthy cow and goat individuals.

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. CH_4 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 CO_2 for available hydrogen, but also provides a nitrogen source for microbial protein synthesis by means of its reduction product ammonia. As shown in Table 2, addition of 10 mM nitrate almost completely inhibited methane synthesis and significantly increased NH_3-N. However, it also brought disadvantages by sharply decreasing the digestibility of ruminal DM, NDF and ADF, fermentation efficiency, and concentration of total VFA, propionate, butyrate, isobutyrate, valerate and isovalerate ($P < 0.01$).

The biggest limitation of nitrate supplementation in ruminant is the accumulation of nitrite, which is highly toxic to many ruminal bacteria and inhibits their metabolic function (Marais *et al*., 1988). The change trend of nitrate and nitrite concentration in rumen liquid fermentation in vitro was presented in Fig. 5. In the two groups without supplemented nitrate, the background level of NO_3^- and NO_2^- was only 0.00–0.30 mM. When nitrate was added, the accumulation of NO_2^- increased with nitrate decrease and reached the highest concentration (3.5 mM) at 12 h. As expected, probably due to the effective nitrite removal ability of *P. aeruginosa*, the nitrite accumulation was significantly decreased at any sampling time and eliminated completely at the end when nitrate supplemented together with *P. aeruginosa* NWAFUP1 strain compared to nitrate supplemented alone ($P < 0.001$).

The supplemented NWAFUP1 strain not only increased the digestibility of DM, NDF and ADF, but also had a synergistic effect with nitrate (Table 1, $P < 0.05$). In addition, it was worth noting that the addition of the NWAFUP1 strain alone significantly decreased 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 product ($P < 0.001$).

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 NH_3-N (Table 2). It meant that less hydrogen was used for reducing nitrate and nitrite to NH_3-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-

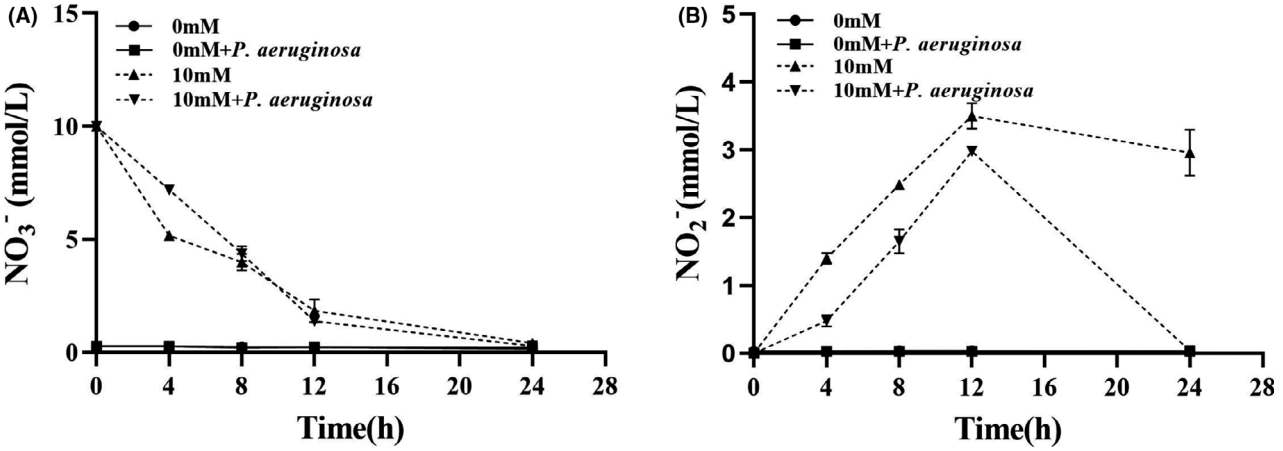

Fig. 5. Effects of adding nitrate alone, the *Pseudomonas aeruginosa* NWAFUP1 strain alone or both of it to rumen fluid on nitrate concentration (A) and nitrite concentration (B). Nitrate effect ($P < 0.001$), *Pseudomonas aeruginosa* NWAFUP1 strain effect ($P < 0.001$) and time effect ($P < 0.001$). Error bars represent standard deviations.

Table 1. Effects of supplement KNO_3 alone or with *P. aeruginosa* NWAFUP1 strain to rumen fluid on the degradation rate of DM, NDF and ADF in the feed.

	KNO_3(–)		KNO_3(+)		*P*-value		
Products produced (%)	*P. aeruginosa* (–)	*P. aeruginosa* (+)	*P. aeruginosa* (–)	*P. aeruginosa* (+)	N	P	N x P
DMD	52.42 ± 1.87	54.41 ± 1.83	47.11 ± 0.94	51.05 ± 4.46	0.021	0.086	0.537
NDFD	36.58 ± 0.52	40.20 ± 3.14	27.45 ± 2.27	38.07 ± 3.26	0.005	0.001	0.044
ADFD	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 KNO_3 alone or with *P. aeruginosa* NWAFUP1 strain to rumen fluid on fermentation characters.

	0 mM		10 mM		*P*-value		
Products produced	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
NH_3-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

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

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 CH_4 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 Purification 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 bioengineering (Shanghai) Co., Ltd. The sequences were blasted for comparing the sequence similarity of the amplified fragments with the 16S rDNA sequence of the predominated 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 NH_4Cl (negative control), 4 mM NH_4Cl (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% $^{13}CH_4$ (Sigma-Aldrich, St. Louis, MO, USA, 99 atm % ^{13}C) and 85% N_2 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 $^{12}CH_4$ (Sigma-Aldrich, 99 atm % ^{12}C) and 85% N_2 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 $\delta^{13}C$-CO_2 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 $^{13}CH_4$ was injected in the isotope group, and 96 ml $^{12}CH_4$ 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 $\delta^{13}C$-CO_2 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-KNO_3 (^{15}N-KNO_3 in the tracer group, and ^{14}N-KNO_3 in the control group). After oxygen removed by repeatedly injecting 99.99% N_2 and vacuum pumping, 96 ml $^{13}CH_4$ was injected into the bottles of the tracer group, and 96 ml $^{12}CH_4$ 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 ^{13}C-CO_2 and ^{15}N-N_2. The concentrations of NO_3^- and NO_2^- 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-$NaNO_2$ (^{15}N-$NaNO_2$ in the tracer group, and ^{14}N-$NaNO_2$ 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 millilitres 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 5×10^7 CFU ml^{-1}), the KNO_3 group (10 mM), and the *P. aeruginosa* NWAFUP1 strain (final concentration of *P. aeruginosa* NWAFUP1 strain was 5×10^7 CFU ml^{-1}) + KNO_3 (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'-CTAYGGRRBGCWGCAG-3') and 806-R (5'-GGACTACNNGGGTATCTAAT-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 (Tanja 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-coverage, 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 ^{13}C-CO_2 and ^{15}N-N_2 was determined by gas chromatography–combustion/thermal conversion–isotope ratio mass spectrometry (GC-C/TCIII-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), NH_3-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 NH_3-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 CH_4 was calculated from gas production.

Statistical analysis

The $\delta^{13}C$-CO_2 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 $\delta^{13}C$-CO_2 and $\delta^{15}N$-N_2 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 NO_3^- and NO_2^- concentration by using two-way repeated measures ANOVA. In brief, each time point was the repeated factor, the KNO_3 and the NWAFUP1 strain were the double factors to observe the dynamic changes of NO_3^- concentration and NO_2^- concentration. Other data, such as CH_4, 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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Supporting information

Additional supporting information may be found online in the Supporting Information section at the end of the article.

Fig. S1. The concentration of NO_3^- and the concentration of NO_3^- was consumed or generated the *P. aeruginosa* NWAFUP1 strain. (A) the *P. aeruginosa* NWAFUP1 strain was cultured in $^{12}CH_4$ and $K^{14}NO_3$ or $^{3}CH^4$ and $K^{15}NO_3$. (B) the *P. aeruginosa* NWAFUP1 was cultured in $^{12}CH_4$ and $Na^{14}NO_2$ or $^{13}CH^4$ and $Na^{15}NO_2$.

Table S1. The sequence data and biodiversity of the two samples in the nitrate-dependent methanotrophs enrichment cultivation system.

Table S2. The sequence data in the nitrate-dependent methanotrophs enrichment cultivation system.

Table S3. Information of qPCR primers.

