The potential of nitrate supplementation for modulating the fermentation pattern and mitigating methane emission in ruminants: A meta-analysis from in vitro experiments

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Abstract. The aim of this study was to evaluate the potential of nitrate supplementation as an in vitro feed additive for modulating the rumen fermentation pattern and mitigating the enteric methane emission by using a meta-analysis method. A database was built from the previously published articles regarding the effectiveness of nitrate as a feed additive in the in vitro rumen fermentation system. Different doses or forms of nitrate supplementations were identified in the database. A total of thirteen studies containing 47 data sets were obtained from ten published research papers. The obtained data were subjected to the mixed model methodology. The doses or the different forms of nitrate were treated as a fixed factor, while the different studies were considered as a random effect. Results showed that nitrate addition decreased significantly (P<0.05) the total gas production, methane production, the TVFAs, and the acetic acid, and increased significantly (P<0.05) ammonia concentration in a linear pattern. However, nitrate did not affect significantly the rumen pH and the population of methanogenic archaea. In conclusion, nitrate is an effective additive for modulating the rumen fermentation by altering the fermentation process resulting in a lower methane production.

1. Introduction

Alongside with the environmental concerns of the methane as a greenhouse gas, methane has potential effects on the feed utilization as well as the animal productivity [1,2]. Methane was considered to be a second greenhouse gas after the carbon dioxide [3]. In addition, methane was reported to be responsible for about 2–12% energy losses in the ruminants [4]. However, it is proposed that, the livestock sector among the other agricultural sectors has caused about 40% of the global greenhouse emissions [5]. As a result of the Kyoto agreement in 1998 in Japan, the agreement aimed to reduce the greenhouse emission to avoid the negative impacts of methane on the environment [6]. Therefore, many countries have adopted the findings of that agreement by constructing strategical plans for mitigating the emission of the greenhouse gases. Further, the animal nutrition scientists also have previously investigated many nutritional approaches for mitigating methane emission in the ruminants [7]. Furthermore, the nutritional
trials were proposed to increase the feed utilization therefore their effectiveness in mitigating the enteric methane emission in the ruminants without any financial increments [8].

Since the rumen methanogens scavenge the H₂ and the CO₂ which produced by the other rumen fermentative microorganisms in the rumen, the mitigation of the enteric methane emission in the ruminants can be achieved by inhibiting directly the rumen methanogens or by altering the metabolic pathway which resulting in a potential reduction of the methanogenesis rate during the fermentation process [5,9]. However, the plant secondary compounds such as tannin and saponin, the supplementation of lipids, or the supplementation of the organic acids was reported to be used effectively for reducing the enteric methane emission in the ruminant animals [5]. However, the potential of nitrate as methane inhibitor is not because nitrate acts as hydrogen sink competing with methanogens on the available hydrogen iron in the rumen resulting in low methane production, the direct inhibition action of the nitrate on the archaeal methanogenic enzyme is another factor for reducing the methane production [10,11] (The toxic property of the nitrate was reported to eliminate the number of the methanogenic bacteria in the rumen as well as the methane production). Despite the effectiveness of nitrate supplementation as a feed additive for modulating the rumen fermentation pattern and mitigating the methane emission in the ruminants, the inclusion of nitrate into the diet is still limited because of the rapid accumulation of nitrite in the blood stream resulting in the methemoglobinemia [12]. However, according to Siclair et al. [13] the partial substitution of the soybeans with NPN can be achieved successfully without interfering the feed digestion or the milk production. Therefore, we aimed in the present meta-analysis work to participate in the debate concerning the potential of nitrate addition, as a feed additive on the modulation the rumen fermentation process and the mitigating the enteric methane emission in the ruminants.

2. Materials and methods

2.1. Database building

In this study, the database was developed from the previously published papers concerning the addition of different doses and forms of nitrate as an in vitro feed additive for modulating the rumen fermentation pattern and mitigating the enteric methane in the ruminants. In this study, the articles those used for building the database were searched using the research engine tools such as Google and Google Scholar and the key words were “nitrate”, “in vitro”, “methane emission” “fermentation pattern” and “ruminants”. The articles were accepted to be used for the database building if the paper contains nitrate and in vitro methane production, nitrate and in vitro or in vivo fermentation pattern, or both nitrate and in vitro methane production and nitrate and the rumen fermentation pattern. If the article contains nitrate beside the any other methane inhibitors in combination or individually, this article did use for developing the development of the database. If the paper involved both the in vivo or in vitro addition of nitrate as a feed additive, the in vitro experiments which involve nitrate and the control diet only are used in the database.

The parameters were used for the development of the database were the total gas production, methane production, the rumen pH value, ammonia concentration, the concentration of the total volatile fatty acids, the portion of the partial volatile fatty acids (C₂, C₃, C₄, IsoC₄, C₅, IsoC₂, and C₂: C₃), the concentration of the nitrate, the concentration of the nitrite, and the methanogenic bacteria. After searching the above key words, we found 25 articles. The next step we evaluated concerning the tittle and the abstract’s content of the collected journals. According to the abstract and the tittle evaluation, we found 20 journals could potentially be used as a source for the development of our meta-analysis database. Then the next stage we carried out a deep evaluation concerning the entire articles. We found only ten journals those could be potentially used for structuring our meta-analysis database. The accepted articles for the database structuring were presented in the Table 1. The entire evaluation of the accepted papers was carried out regarding the addition of nitrate as an in vitro feed additive, whether for methane mitigation or for the manipulation of the fermentation pattern.
As it is summarized in the Table 1, the addition of the nitrate as in vitro feed additive was carried out using different doses and forms of nitrate in the diet (0g/kg to 17.60g/kg diet). Nitrate was added, starting from 0% (control diet) until 20 g/kg diet as in highest addition in the collected papers those were used in the database. It is shown also, in the table No (1), that the different nitrate forms were supplemented for mitigating methane emission and manipulating the fermentation pattern. The nitrate forms were categorized into main groups, nitrate individually (NO₃) and the nitrate salt. The salt contains sodium nitrate; potassium nitrate, sodium nitrite, and the ammonium nitrate. Urea addition was excluded from the dataset that is used for the database. All the components in the category which content nitrate or nitrite were classified as (Nitrate salt). In case of the compounds those contain nitrate or nitrate in a salt form, the calculation of the nitrate content was done as the molar mass based on the percentage of nitrate that involved in those compounds.

2.2. The data analysis

The data included in the database were measured using compliable measurement units and further were analyzed using the statistical meta-analysis based on the mixed model in accordance with Hidayat et al. [14]. The different studies were grouped as random effects while the different doses or forms of nitrate were analyzed as fixed effects. To determine the predictor variables, whether continuous or discrete, the present meta-analysis work used two statistical models. The statistical model that we used was based on the P-values. We used p-values (p<0.05) as a criterion for determining the significant effects of each variable. When the p-value was between 0.05 and 0.1, this indicates that the effect had a tendency to be significant. In this study, all the statistical analyses were preceded using SAS software version. 9.4.

### Table 1. The studies those are involved in structuring the meta-analysis database

| No | References | Number of studies | Period (hour) | Nitrate form | Dose (g/kg) |
|----|------------|-------------------|---------------|--------------|------------|
| 1  | Zhao et al. [5] | 1 | 24 | (Nitrate) | (0 – 4.8) |
| 2  | Lee et al. [13] | 1 | 24 | (Urea, nitrate, and encapsulated nitrate) | (0.08 – 0.09) |
| 3  | Mamvura et al. [15] | 1 | 24 | (Nitrate, encapsulated nitrate) | (0 – 1.785) |
| 4  | Asanuma et al. [16] | 1 | 24 | (Potassium nitrate) | (0 – 6.88) |
| 5  | Lund et al. [20] | 1 | 48 | (Nitrate) | (0 – 20) |
| 6  | Nguyen et al. [24] | 2 | 24 | (Sodium Nitrate) | (0 – 14.588) |
| 7  | Zhou et al. [35] | 2 | 48 | (Nitrate) | (1.02) |
| 8  | Bachruddin et al. [36] | 1 | 48 | (Nitrate) | (0 – 0.465) |
| 9  | Natel et al. [37] | 2 | 24 | (Nitrate, encapsulated nitrate) | (0 – 2.64) |
| 10 | Sun et al. [38] | 1 | 12 | (Urea, nitrate, and nitrite) | (0 – 0.2) |

3. Results and discussion

In this study, the references those used for developing the database were presented in Table 1. Tables 2, 3 and figures 1, 2, 3, and 4 summarize the effects of nitrate (forms or doses) as an in vitro feed additive on the rumen fermentation characteristics and the enteric methane emission in the rumen. However, the results have shown that both the doses or the forms nitrate did not affect significantly the rumen pH value, the population number of the methanogenic archaea, and the precipitation of the nitrate and the nitric oxide in the rumen in a linear pattern (P<0.05). As compared with the control diet, all the treatments of nitrate (forms or doses) have influenced significantly the total gas production, methane production, the total volatile fatty acids, the concentration of the molar portion of the acetic acid, and the ammonia concentration in the rumen in a linear pattern (P<0.05) Table 2, 3, and figures 1, 2, and 4. The increase of the nitrate doses have decreased significantly the total gas production, methane production the total volatile fatty acids, and the molar portion of the acetic acid in the rumen in a linear pattern, while the lowest methane production, total volatile fatty acid were obtained as a result of adding the nitrate in a salt form. The highest ammonia concentration was due to the inclusion of the nitrate in a salt form.

The supplementation with nitrate (in different doses or forms) did affect significantly the rumen pH value (P<0.05) Tables 1 and 2. As it is reported that the inclusion of both nitrate or the encapsulated
nitrate into the experimental diet did not change the rumen pH value [15]. This indicates that, the inclusion of nitrate individually, nitrate in a salt form, or the encapsulated nitrate do not have any negative impacts on the rumen condition or the fermentation process. The result was in consistence with Asanuma et al. [16]. Normally, the rumen pH value is between (6.4 – 6.8), whereas the rumen pH values between 5.0 and 5.5 are considered abnormal values [17]. The normal pH value always reflects the optimum conditions in the rumen [2]. It was reported that the decrease in the rumen pH value could affect significantly the rumen microorganism composition as well as the ruminal fermentation pattern [18]. This means that, the inclusion of the nitrate in the diet does not harmfully affect the rumen condition or the fermentation process in the ruminant.

### Table 2. Regression equations on the influence of the different doses of nitrate forms on the in vitro rumen fermentation pattern and methane emission in ruminants.

| Response Parameter | Unit | Model | N | Parameter estimates | Model estimates |
|--------------------|------|-------|---|---------------------|-----------------|
|                    |      |       |   | Intercept SE Intercept | Slope SE Slope | P-value | AIC^2 |
| Total gas          | mL   | L     | 27| 137.31 237.19 -1.6919 | 0.4384 0.0012 | 218.2  |
| pH                 |      |       |   | 6.5350 0.09771 0.01786 | 0.00346 0.0001 | -13.6  |
| CH₄                | mM   | L     | 21| 0.9293 0.1791 -0.03005 | 0.08817 0.0028 | 12.8   |
| NH₃                | mM   | L     | 24| 5.2873 1.5896 0.1768 | 0.06411 0.0146 | 114.1  |
| TVFAs              | mM   | L     | 44| 90.7444 9.0121 -1.2084 | 0.3264 0.0008 | 334.8  |
| C₂                 | %    | L     | 44| 64.3524 4.7745 -0.6245 | 0.2095 0.0055 | 300.0  |
| C₃                 | %    | L     | 44| 25.5872 13.2578 0.07439 | 0.3180 0.8166 | 334.6  |
| C₄                 | %    | L     | 44| 3.1006 1.2229 -0.03880 | 0.03184 0.2323 | 143.3  |
| ISOC₄              | %    | L     | 28| 0.2333 0.1125 -0.00241 | 0.003500 0.4989 | -41.1  |
| C₅                 | %    | L     | 28| 0.03986 0.02420 -0.00018 | 0.000254 0.4888 | -155.5 |
| ISO₂C₄             | %    | L     | 28| 0.06801 0.04190 -0.00044 | 0.000527 0.4103 | -99.1  |
| C₂/C₃              | %    | L     | 44| 3.9537 0.3736 0.03790 | 0.05147 0.4671 | 155.7  |
| N₂O                | mM   | L     | 16| 1.1822 0.6101 -0.06492 | 0.05274 0.2440 | 16.6   |
| N₂O₃               | mM   | L     | 13| 1.3143 0.9310 0.004612 | 0.003705 0.2446 | -4.2   |
| Methanogens (%)     |      | L     | 14| 23.4720 22.9672 -0.1217 | 0.1355 0.3928 | 57.9   |

Note: SE; standard error; mM= the concentration in a mil mole, L; the linear regression equation, Q; Quadratic regression equation, AIC; the estimator of the relative quality of statistical models for a given data set, P-value;

### Table 3. The effects of the forms of nitrate on the methane production and the in vitro fermentation characteristics.

| Item            | Dose average | Unit | N | Control | Nitrate form | Salt | p-value |
|-----------------|--------------|------|---|---------|--------------|------|---------|
| Total gas       | mL           | 27   |   | 139.00  | 114.78       | 134.47 | 0.0347  |
| pH              | mM           | 24   |   | 6.5504  | 6.5215       | 6.7017 | 0.0123  |
| CH₄             | mM           | 21   |   | 0.9929  | 0.8542       | 0.2282 | < 0.0001|
| NH₃             | mM           | 24   |   | 4.8618  | 5.1418       | 9.7777 | < 0.0001|
| TVFAs           | mM           | 44   |   | 93.6359 | 90.6784      | 80.3533 | 0.0033  |
| C₂              | %            | 44   |   | 64.0221 | 64.1948      | 60.2308 | 0.3365  |
| C₃              | %            | 4    |   | 23.1197 | 21.4728      | 33.4521 | 0.0277  |
| C₄              | %            | 44   |   | 3.5470  | 2.5064       | 9.7777 | 0.0084  |
| ISO₂C₄          | %            | 28   |   | 0.2731  | 0.2072       | 0.2389 | 0.0924  |
| C₅              | %            | 28   |   | 0.04097 | 0.03991      | 0.03725 | 0.6485  |
| ISO₂C₃          | %            | 28   |   | 0.07053 | 0.06572      | 0.06700 | 0.5839  |
| C₂/C₃           | %            | 44   |   | 3.3758  | 3.7708       | 4.6675 | 0.0661  |
| N₂O₂            | mM           | 16   |   | 1.1449  | 1.0592       | 1.1526 | 0.9439  |
| N₂O₃            | mM           | 13   |   | 1.2700  | 1.3715       | 1.3393 | 0.0822  |
| Methanogens (%) | %            | 14   |   | 23.8346 | 23.5519      | 22.4649 | 0.0912  |

Note: N.Salt; nitrate addition in a salt form (sodium nitrate, potassium nitrate, ammonium nitrate, and sodium nitrite).

The increase of the nitrate dose has d the concentration of the total volatile fatty acid (P<0.05). As compare with control diet, all the nitrate forms have decreased linearly (P<0.05) the concentration of the VFAs, while the lowest concentration of the TVFAs was due to the inclusion of the nitrate in a salt
from. Anderson et al. [19] reported that the inclusion of the nitrate into the diet has decreased the concentration of the TVFAs. Nitrate addition was reported to alter the molar portion of the acetic propionate as well as the butyrate at all incubation times [20]. The precious study of Adejoro and Hassen [21] found that the nitrate tends to decrease both the acetate and the propionate. In this study nitrate has affected only the acetic acid portion, but did not affect the concentration of the acetic: propionic. Adejoro and Hassen [21] has mentioned that the decrease in the molar portion of the acetic acid was higher than the propionic acid portion. Patra et al. [7] reported that nitrate did not influence the concentration of the acetic acid portion.

The concentration of the molar portion of the propionic and the butyric acid did not affect linearly (P <0.05) due to the supplementation with the nitrate [22]. The reduction of the acetic acid causes a reduction in the hydrogen concentration which may later on affect the methanogenesis and methane production.

Figure 1. The effects of nitrate on the methane production

Figure 2. The effects of nitrate on the acetic acid production

Figure 3. The effects of the nitrate on the ammonia concentration

Figure 4. The effects of nitrate on the propionic acid production

The ammonia concentration, however, has increased linearly (P<0.05) by increasing the nitrate dose, while the highest concentration of the ammonia was due to the inclusion of nitrate in a salt form in comparison with the control diet and the diet that was supplemented with the nitrate individually (No3). In addition, Lee et al. [23] reported an increase in the ammonia concentration due to the inclusion of the nitrate into the diet. Similarly Asanuma et al. [16] observed a significant increase of the ammonia concentration after the inclusion of the nitrate into the diet. The increase of the ammonia concentration in the rumen was proposed because of the rapid reduction of nitrate to nitrite and consequently to ammonia [15]. The increase of the ammonia could be followed by high protein synthesis, which with enhance the animal performance because the higher accumulation of the ammonia in the rumen could reflect higher digestion and higher feed utilization. This could improve in the microbial protein synthesis as well as the nitrogen retention [24]. In general, nitrate is safe and can be used in a salt-form or nitrate individually (No3) without any negative effects on the nutrient digestion or ammonia concentration. Therefore, we recommend that the in vivo meta-analysis study concerning the evolution of the feed intake as well as the feed utilization.

As the nitrate doses increases, the total gas production decreases (P<0.05). However, the lowest gas production rate was because of including the nitrate in a salt-form. The decrease of the gas production was stated because the nitrate is toxic for the [22]. Similarly, Getachew et al. [25] and Calabró et al. [26] found that the inclusion of nitrate into the diet decreases the gas production. Gas production indicates the direct activity of the microbes on the fermentation process, while the better index of the gas production can be obtained at the forage ME than fermented nutrient because the carbohydrates are the major gas produced as compared with the other feed ingredients [27]. Therefore, the reduction of
the total gas production does not always reflect the low digestibility and low feed utilization. The gas production rate was observed to be larger at the fed which contains more fibrous substrate [28]. The reduction of the total gas production could be attributed to the suboptimum condition in the rumen, but it could be also because of the low fibrous content in the feed as well. This result was in consistence with van Zijderveld et al. [29] and Guyader et al. [30]. It is proposed that the addition of nitrate into the exponential diets decreased the availability of the hydrogen iron, which eliminates the methanogenesis rate resulting a low methane production [31,32]. Although there was no significant reduction of the rumen methanogens population due to the inclusion of nitrate in the experimental diet, the concentration of the methanogenic bacteria of nitrate treatments were numerically lower than the control diet. As a sequence of the numerical reduction of the methanogens population, methane production is expected be reduced. Zhao et al. [5] has reported a numerical decrease of the population of the rumen methanogens bacteria after nitrate supplementation. The mode of action of the nitrate against the methanogens and the methane production is previously reviewed by many authors [12,33,34]. Therefore, these results indicate the effectiveness of nitrate as a methane inhibitor in the ruminants because of its ability to reduce the acetic acid formation resulting in lower hydrogen production and subsequently lower methane production. This concludes that, nitrate can effectively eliminate the acetic acid production in the rumen and therefore it eliminates the methane production by reducing the hydrogen’s availability for the rumen methanogens.

As compared with the control diet, the different doses or the forms of nitrate the previous studies did not show any significant accumulation of the nitrate or nitric oxide in the rumen. In addition, Getachew et al. [25] reported that the inclusion of the encapsulated nitrate has reduced the accumulation of the nitric oxide in the rumen as compared with the non-encapsulated nitrate addition. The high accumulation of the nitric oxide is reported to cause nitrate toxicity because its linked with the blood Methemoglobinemia in the ruminants [35]. The high accumulation of the nitric oxide could be produced chiefly because of the high inclusion of the nitrate into the diet in the ruminant. The excessive dose of nitrate rate express clinical sings regarding the nitrate toxicity, which is expected to appear at the high addition of nitrate levels into the diet [35]. It proposed that the nitric oxide is considered to be an intermediate product, which is rapidly converted into ammonia [36]. To our knowledge, the conversion of the nitric oxide into ammonia could bring nutritious benefits, especially in the ruminant because it is linked to the microbial protein synthesis in the rumen. Therefore, the optimum dose of nitrate should not be exceeded the maximum toxic ranges to aid the nitrate to be utilized effective way without any adverse effects.

4. Conclusion
In conclusion, the inclusion of nitrate into the experimental diets is effective in modulating the fermentation process in the rumen by reducing the concentration of the total gas production, the total volatile fatty acids, and the molar portion of the acetic acid resulting in a lower methane production. However, the effectiveness of nitrate supplementation on mitigating methane emission in the rumen could be because of the direct effects of nitrate on the acetic acid formation which influence negatively the methanogenesis process resulting in lower methane production. The inclusion of nitrate in a salt form is more effective than nitrate individually for mitigating the enteric methane emission in the ruminants. Therefore, the inclusion of the nitrate into the in vitro experimental diets is recommended to be used safely without any adverse effects on the ruminants’ animals. Furthermore, the in vivo meta-analysis work is recommended to be carried out.

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