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Magnesium in dairy cattle nutrition: A meta-analysis on magnesium absorption in dairy cattle and assessment of simple solubility tests to predict magnesium availability from supplemental sources

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ABSTRACT

Supplemental Mg sources differ in bioavailability, and solubility is one of the determining factors. We explored whether and which in vitro solubility tests could reliably differentiate the quality of supplemental Mg sources. In experiment 1, we compared 3 chemical methods using an acetic acid solution (50 mL/L, termed vinegar test), a 1 *M* ammonium nitrate solution, and an artificial rumen buffer fluid without rumen microbiota. The Mg solubility results suggested the vinegar test was the best method due to its robustness, simplicity, and reproducibility. In experiment 2, we validated the reliability of the vinegar test using 4 MgO sources from experiment 1 and 12 new MgO sources plus a laboratory-grade MgO as a standard. Accordingly, we repeated the vinegar test with short (0.5 h) and long (3.0 h) incubation times on these sources and then conducted ruminal incubations in 24-h batch culture experiments. The repeated vinegar test resulted in similar results as in experiment 1. Linear regression across both experiments showed the soluble Mg content (g/kg) = 44.46 (±2.55) × pH − 142.9 (±14.9), root mean square error (RMSE) = 10.2, *P* slope <0.001, and concordance correlation coefficient (CCC) = 0.953. The predictable pH range was from 4 to 6. The equation cannot be applied to low-alkaline sources such as Mg sulfate, Mg acetate, or a group of MgO with exceptionally high alkaline properties showing a cluster of pH above 8.5. Solubility of the MgO sources in the vinegar test ranged from 5 to 35%, whereas the 24-h ruminal incubations led to more solubility (15–70%). Nevertheless, the differences among most MgO sources were parallel to the data from the in vitro rumen solubility. Next, we performed a meta-analysis of published studies (21 studies, 94 treatments) to assess the true Mg absorption in vivo and potential factors affecting Mg absorption in dairy cows. It appeared that on average dairy cows absorbed about 20% of the Mg intake (range 10–40%), regardless of their lactation status. We revealed a new strategy to predict Mg absorption relative to dietary K as follows: true Mg absorption (g/d) = 0.3395 (±0.025, $P < 0.001$) × Mg intake (g/d) − 1.9273 (±1.16, $P = 0.11$) when dietary K ≤20 g/kg DM, and 0.154 (±1.06, $P = 0.05$) + 0.209 (±0.026, $P < 0.001$) × Mg intake (g/d) when dietary K >20 g/kg DM (RMSE = 2.19). This strategy improved the accuracy of prediction as compared with the existing prediction (CCC = 0.922 vs. 0.845). Still, over- or underestimations inherent to individual studies were evident and might be related to unaccountable factors, especially the quality of supplemental Mg sources. In conclusion, the vinegar test is a useful tool to rank inorganic Mg sources with alkaline properties. Including in vitro solubility data in Mg nutrition research could help to refine the prediction of bioavailable Mg contents and increase precision in feed formulation.
Key words: magnesium oxide, mineral, vinegar test, ammonium nitrate, ruminant

INTRODUCTION

Magnesium is an essential mineral required for many vital processes of the body and is one of the most critical macro-minerals in dairy cows. Magnesium is mainly absorbed in the rumen, and high K levels significantly impair its uptake (Suttle, 2010; Goff, 2014). Notably, Mg deficiency cannot be readily compensated by the Mg stores in the bone (Martens and Stumpff, 2019). The level of Mg in milk is kept constant by draining Mg from the blood pool regardless of the intake (Laporte-Uribe, 2005). When Mg is undersupplied, animals develop various degrees of hypomagnesemia that could be fatal. Typical feedstuffs for ruminants do not always provide adequate Mg supply. For instance,

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milk for calf rearing as well as plants such as spring grasses, corn silage, and cereal grains are marginal in Mg contents, with grasses being also rich in K (Suttle, 2010). Legume forages and oilseed meals are richer in Mg (Suttle, 2010). However, because of their restricted use in the diet, their contribution often is not enough to meet Mg requirements in milk-fed calves, pasture-fed ruminants, or high-producing ruminants. Thus, inorganic Mg sources are typically added to dairy rations to secure adequate daily Mg intake in such cases. However, inorganic Mg sources used as feed supplements for ruminants are distinctively different in their Mg content as well as in the bioavailability of Mg. Magnesium oxide (**MgO**) is a more popular choice of Mg supplements because it typically contains relatively high Mg contents (50–60%) and is more palatable than magnesium sulfate and magnesium acetate. Magnesium oxide provides an alkaline property; hence it is also used as an antacid agent in the rumen when feeding high-grain diets (Beede, 2017). High Mg contents, however, do not necessarily guarantee high bioavailability of the Mg sources because Mg bioavailability depends on the solubility, absorbability, as well as reactivity of the Mg with other molecules or compounds in the rumen (Suttle, 2010; Goff, 2014). Large variations already exist in quality among MgO sources associated with geographical origins and calcination temperatures of ores (Beede, 2017).

Finding a rapid yet reliable method to compare inorganic supplemental sources is highly beneficial for diet formulation. A selection of the sources with high ruminal availability will help to reduce the inclusion levels of raw materials in the ration and decrease losses of the unavailable minerals into the environment. No gold standard currently exists for a laboratory method to determine the availability of Mg sources. However, in vitro solubility tests could be an indicator, considering that the solubility of Mg is a prerequisite to ruminal absorption (Dalley et al., 1997; Goff, 2014). A recent study has also pointed out the necessity of developing a standardized procedure for solubility tests for use in calculating digestible Mg for ruminant diets (Martens and Stumpff, 2019). In literature, in vitro tests include use of acidic solutions such as hydrochloric acid (Xin et al., 1989), citric acid (Schonewille et al., 1992), acetic acid (Goff, 2014), and ammonium nitrate (Tsiplakou et al., 2017) to test the solubility of inorganic Mg sources, especially for MgO. These simple tests, however, lack organic components in the rumen, particularly ruminal microbiota, which can affect the solubility of Mg (Lough et al., 1990). Some studies have shown that the ranking of Mg sources based on the in vitro solubility is parallel to the in vivo solubility (Tsiplakou et al., 2017) or ruminal Mg concentrations (Schonewille et al., 1992). However, studies have employed different tests and mainly tested a small set of MgO sources. No clear conclusion has been reached for the performance and implications of the available in vitro solubility methods in screening inorganic Mg sources.

We hypothesized that in vitro solubility tests can reveal the potentially available Mg contents of the inorganic supplemental sources, but a certain method might be superior to the others. The present study aimed to reveal the most promising chemical method, among tested methods, for screening supplemental Mg sources as well as to examine its implications for practical use. To do so, we compared 3 known Mg solubility tests using a vinegar solution (Goff, 2014) and an ammonium nitrate solution (Tsiplakou et al., 2017), and a modified artificial ruminal fluid (Bales et al., 1976), which was closer in resembling rumen conditions compared with the acid tests. We performed 2 independent experiments for in vitro solubility tests, the first to identify the best method and the second to test the reproducibility of the chosen method using an independent set of Mg sources. We further validated the chosen method under in vitro rumen conditions in batch culture experiments. More than 20 Mg sources were tested across all experiments. With this amount of data, we expected to develop an accurate equation for predicting soluble Mg contents via the pH from the in vitro solubility data, underlining the practical implication of the method. Such predictions would be highly valuable for nutritionists and feed mills that have no facility for Mg analysis. Finally, we performed a meta-analysis of published studies to determine the absorption values of Mg in vivo as another measure of the in vitro solubility data, expecting that a promising in vitro method would show solubility values that fall within the in vivo range while keeping in mind the influential factors beyond the solubility. Research has pointed out the negative role and mechanisms of dietary K interfering with ruminal Mg absorption (Schonewille et al., 2008; Martens and Stumpff, 2019). However, this was not unanimously observed (Holtenius et al., 2008). Schonewille et al. (2008) conducted a meta-analysis to predict Mg absorption in dairy cows. The outcome was derived from studies with the majority using dry cows and byproduct-based diets containing high K contents. Therefore, we updated the database including newer studies and performed a meta-analysis to reveal Mg absorption in dairy cattle with an emphasis on dietary K content and lactation status that could influence the absorption of otherwise solubilized Mg (Martens and Stumpff, 2019).

MATERIALS AND METHODS

Because no human or animal subjects were used, this analysis did not require approval by an Institutional Animal Care and Use Committee or Institutional Review Board.

In Vitro Experiments

We performed 2 independent experiments in 2019 (experiment 1) and then in 2022 (experiment 2) in the present study. Experiment 1 served to screen the most promising solubility test. We used various kinds of Mg sources and 3 different in vitro solubility tests. Experiment 2 was performed to validate the chosen method with an emphasis on MgO sources.

Mg Sources. In experiment 1, 15 Mg sources containing low to high Mg contents (3.7–557.8 g/kg as analyzed) were used in the present study (Table 1). Of these, 8 were MgO sources (named alphabetically from MgO-A to MgO-H), 2 magnesium phosphate sources (Mg phosphate-A, Mg phosphate-B), 1 magnesium acetate source (Mg acetate), and 3 clay minerals (clinoptilolite, bentonite, sepiolite). In general, MgO sources (except MgO-E) contained higher contents of Mg, about twice as much of Mg phosphate and Mg sulfate, and almost 3 times that of Mg acetate. The clay minerals were generally low in Mg content.

In experiment 2, we studied 4 MgO sources (MgO-A to -D) from experiment 1 and 12 new MgO sources from a different feed plant and a laboratory-grade MgO (as standard). Some MgO samples were from the same source but had different particle sizes (MgO-I and -J; MgO-K, -L, and -M; and MgO-P, -Q, and -R). The cumulative particle size distribution of these MgO samples is shown in Figure 1.

In Vitro Solubility Tests (Experiment 1). The study was carried out at Nuscience, Drongen, Belgium. The first test was the vinegar test, performed according to Goff (2014), which was the focus of the present study because it has been recommended by previous research (Beede, 2017; Martens and Stumpff, 2019). Exactly 3.0 g of each Mg source was placed in a container, and 40 mL of an acetic acid solution (50 mL/L, pH 2.45) was added. The container was closed and shaken for 15 s, let sit, and shaken again after 15 min of incubation. Subsequently, the pH of the solution was measured. Each Mg source was tested in quadruplicate. The original method stated 0.5 h as the incubation time; we also evaluated multiple time points to determine the time-dependent performance of the method. We tested Mg sources in 3 batches. The first batch of samples consisted of 9 sources, 2 of which were MgO sources (MgO-A and -B; Table 1). These were used for detailed observations for pH changes with incubation time. For that, we measured the pH at several time points (0.5, 1, 3, 24, and 48 h), and the final 48-h samples were taken for analysis of the soluble Mg content. The second batch contained samples of 6 MgO sources (MgO-C to -H, Table 1). They were subjected to 0.5-h and 3-h time point measurements, and the final 3-h samples were used for Mg analysis. Then we selected 6 sources including 3 MgO sources (MgO-A, -C, and -D), Mg phosphate-A, Mg sulfate, and sepiolite, and repeated the vinegar test with 0.5-h and 24-h time points (batch 3). The selected MgO sources were from the same supplier but with different geographic origins. The 24-h data were used for comparisons with the other 2 methods that used the same test duration.

The second test, termed the NH_4NO_3 test, was performed according to Tsiplakou et al. (2017). Briefly, exactly 1.0 g of each Mg source was mixed with 200 mL of 1 *M* ammonium nitrate solution (pH of 4.79), shaken for 15 s, and then kept at 39°C for 24 h with occasional stirring. After cooling to ambient temperature, the pH of the solution was measured and subsequently taken

Table 1. Tested Mg sources[1] and the analyzed Mg content of Mg sources used for in vitro experiments

Experiment no.[2]	Mg sources	Mg content (g/kg)
1	Mg sulfate anhydrous	211.3
1	Mg phosphate-A	242.7
1	Mg phosphate-B	257.8
1	Mg acetate	171.1
1	Clinoptilolite	3.7
1	Bentonite	14.5
1	Sepiolite	106.5
1 and 2	MgO-A	557.0
1 and 2	MgO-B	546.6
1 and 2	MgO-C	484.3
1 and 2	MgO-D	522.5
1	MgO-E	272.0
1	MgO-F	524.8
1	MgO-G	514.5
1	MgO-H	555.4
2	MgO-I	475.0
2	MgO-J	482.3
2	MgO-K	499.6
2	MgO-L	495.1
2	MgO-M	500.5
2	MgO-N	492.1
2	MgO-O	415.5
2	MgO-P	506.5
2	MgO-Q	516.5
2	MgO-R	520.5
2	MgO-S	490.1
2	MgO-T	464.4
2	MgO standard	510.0

[1]MgO = magnesium oxide, Mg sulfate = magnesium sulfate, Mg phosphate = magnesium phosphate. Letters A through T indicate magnesium sources differing in geographical origins, manufacturers, and particle sizes.

[2]Mg sources used for experiment 1 were from a feed manufacturer in Belgium and for experiment 2 from a feed manufacturer in Austria.

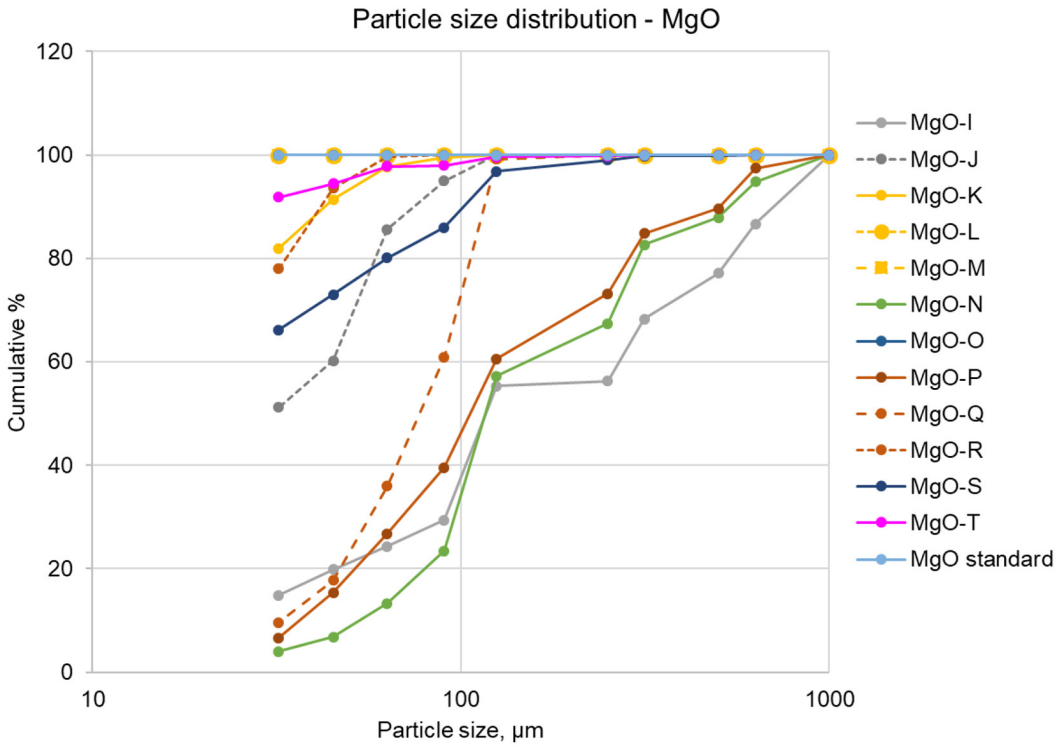

Figure 1. Accumulative particle distribution of magnesium oxides (experiment 2; see Table 1). Sources sharing the color theme (e.g., MgO-P, -Q, and -R) are the same source but differ in particle size. Samples were sorted through a series of sieves with pore sizes of 1,000, 630, 500, 315, 250, 125, 90, 63, 45, and 32 µm, respectively. The smaller the particles, the accumulative contents reach 100% at smaller sieve sizes. For instance, MgO-R contained 80% of particles smaller than 32 µm, whereas less than 10% of MgO-P and Q were smaller than 32 µm. MgO-L and MgO standard had the finest particles, with all particles smaller than 32 µm.

for Mg analysis. Soluble Mg is exchangeable with NH_3^+, thereby raising the pH of the solution. Again, each Mg source was tested in quadruplicate.

The last test, termed the artificial ruminal fluid (**ARF**) test, was performed by mixing 1.0 g of each Mg source with 200 mL of artificial ruminal fluid prepared according to Bales et al. (1976) but without the addition of rumen inoculum. The artificial rumen fluid contained (g/L) 2.86 acetic acid, 0.113 valeric acid, 0.058 isobutyric acid, 0.75 urea, 1.57 HCl, 0.82 $(NH4)_3PO_4$, 0.227 CaCl2, 0.25 $(NH_4)_2SO_4$, 0.20 $MgSO_4$, 13.60 $NaHCO_3$, 0.66 KCl, 1.0 casein, 0.50 Cys, 0.00005 biotin, 0.0001 para-aminobenzoic acid, and 0.0015 $CoCl_2$. The final solution was adjusted with HCl to a pH of 6.5 before use. The mixture was incubated at 39°C for 24 h with occasional stirring. After cooling to ambient temperature, the pH of the solution was measured and subsequently taken for Mg analysis. Again, each Mg source was tested in quadruplicate.

Repeated Vinegar Test and In Vitro Rumen Incubation (Experiment 2). Each of the MgO sources used in experiment 2 (Table 1) was subjected to the vinegar test using the protocol as described previously with 2 incubation time points of 0.5 and 3 h. The test was carried out at the Institute of Animal Nutrition and Functional Plant Compounds, Vetmeduni, Vienna. The liquid samples were stored at −20°C for later analysis of Mg contents.

A total of 15 MgO sources were used for 24-h incubation with rumen fluid inoculum using the Hohenheim gas test (Menke and Steingass, 1988). The anaerobic incubation was carried out using gas-tight glass syringes, which were kept in a chamber regulated at 39°C throughout the trial. Two independent batch culture runs were performed. The rumen fluid inoculum was obtained from a rumen-cannulated cow for each batch culture experiment. Both donor cows were nonpregnant and nonproducing. They were fed mainly hay and a daily allowance of 0.5 kg of commercial concentrate. The donor cows were kept according to Austrian guidelines (114) for animal welfare (BGBl. II Nr. 485/2004 idF BGBl. II Nr. 151/2017). Each MgO source was tested in duplicate in each batch culture experiment (n = 4 per treatment in total). Treatments were randomly assigned to syringes and the order of treatments was subject to inoculation, placement in the chamber, as well as at the termination changed between the experiments, to prevent bias related to time among

treatments. In addition, blank and unsupplemented diet units were also included to account for soluble Mg coming from unsupplemented sources (buffer inoculum and feedstock). The feedstock providing substrates for rumen microbial fermentation was a mixed diet for dairy cows with a forage-to-concentrate ratio of 50:50 on a DM basis. The diet contained, on a DM basis, 92.8% OM, 17.0% CP, 1.80 ether extract, 7.20% ash, and 39.2% NDF. Each incubation unit was incubated with 200 mg DM feedstock and for MgO treatments also with 15 mg of each MgO. Each incubation unit was subsequently inoculated with 30 mL of prewarmed rumen fluid-buffer solution. The rumen fluid buffer solution was prepared according to Menke and Steingass (1988). In each batch culture run, triplicates of blanks containing only the rumen fluid buffer solution were included. After 24 h, the incubation was halted and the incubation fluid was centrifuged in 2 steps, starting at 3,220 × *g* for 15 min; then the supernatant was collected and centrifuged at 23,640 × *g* for 15 min. The final supernatant (6 mL) was acidified with 0.9 mL of 0.5 *N* HCl to keep the pH <5.0 before storage at −20°C for later analysis of Mg content.

Mg Analysis

Original Mg samples and solutions after incubation were used for Mg analysis using inductively coupled plasma optical emission spectroscopy (**ICP-OES**; Avio 500, PerkinElmer) to determine the total Mg and soluble Mg content, respectively. For original Mg materials, ~1.0 g of each source was hydrolyzed with 40 mL of 6 *N* HCl boiling for 1 h and then diluted with deionized water and filtrated to 1,000 mL. Next, 0.5 mL of the solution was mixed with 9.5 mL of 0.5 *N* HCl before the ICP-OES analysis. For soluble Mg contents, the liquid samples were centrifuged at 13,793 × *g* for 10 min to remove any solid particles, and the supernatant was collected. Before ICP-OES analysis, the supernatant was diluted with 0.5 *N* HCl. Samples of Mg sources with low Mg contents were treated with a lower dilution rate. The Mg contents were calculated from standards and dilution rates. For batch culture experiments, the soluble Mg content derived from the added MgO was estimated from the differences between MgO-supplemented treatments and diet alone. Solubility of Mg (percentage) was calculated from the soluble content relative to total Mg content × 100.

Meta-Analysis

We performed a meta-analysis to evaluate the in vivo availability of Mg based on the true absorption as an indirect means to evaluate whether the in vitro solubility tests show values within the logical range of in vivo values. Additionally, we also determined dietary and cow factors that likely affect the true absorption of Mg. It must be noted that Schonewille et al. (2008) already performed such a meta-analysis and reported prediction equations for Mg absorption in dairy cows. However, their database was derived from experiments conducted decades ago (1961–2004). Importantly, several of these experiments used dry cows, and many studies were from the same group, thus using similar dietary formulations and feeding. Findings of newer studies disagree with the previous suggestion—for instance, Holtenius et al. (2008) versus Jittakhot et al. (2004c)—despite performing identical treatment plans. Therefore, we have updated the work done by Schonewille et al. (2008). Web of Science (https://www.webofscience.com/wos/woscc/basic-search) was used for the literature search, using key word terms such as magnesium, magnesium absorption, mineral absorption, dairy cattle, and dairy cows. The search was limited to original research articles, full-text accessible, published between the years 1961 and present, and in the following Web of Science categories: Agriculture Dairy Animal Science, Veterinary Science, Agriculture Multidisciplinary, and Biology. For each search performed, the resulting articles were screened to meet all criteria, including (1) works done in dairy cattle, (2) reporting lactation status and average BW of the animals, (3) reporting or allowing calculation for dietary K and Mg, (4) reporting or allowing calculation for daily K and Mg intake, and (5) reporting or allowing calculation for fecal Mg outputs. One treatment from Ben-Ghedalia et al. (1996) was excluded from the database because the treatment used poultry litter as the source of minerals. The final database used in the present study consisted of 21 studies with 94 dietary treatments, with 6 new studies (26 treatments) added to the existing database reported in Schonewille et al. (2008). The process of literature search and study collection (i.e., the PRISMA flowchart as outlined by Page et al., 2021) is visualized in Supplemental Figure S1, and the database is presented in Supplemental Table S1 (https://data.mendeley.com/datasets/9v85ry8t3s/2). True absorption of Mg (g/d) was estimated from the apparent absorption (g/d) + endogenous Mg secretion (g/d). The endogenous Mg secretion was calculated as follows: 0.004 × BW (Schonewille et al., 2008). Extending the work done by Schonewille et al. (2008), we characterized the responses in relation to lactation status (dry cows vs. lactating cows) in addition to the effect of dietary K on Mg absorption. One study (Schonewille et al., 1994a) used pregnant heifers. We placed the data in the dry cow category in the present study. Identifying the influence of dietary K on true Mg absorption was performed in 2 ways: as a quantitative predictor and as

a discrete predictor. The latter approach was derived from the newer data revealing different responses depending on diet K level relative to the Mg intake. For this, the dietary treatments were categorized as low K when the dietary K level was ≤20 g/kg DM and high K when the level was >20 g/kg DM.

Statistical Analysis

All data were analyzed using the MIXED procedure of SAS (version 9.4., SAS Institute Inc., Cary, NC), unless otherwise stated. Magnesium analysis was performed in different blocks; this factor was included as a random effect in the mixed model for data analysis of Mg concentration and solubility. For experiment 1, data on the pH of the vinegar solution measured at different incubation time points were analyzed as repeated measures of time, testing the fixed effects of Mg source, time, and their interaction. Data from selected Mg sources at 24 h of incubation were used to compare the method and the statistical model, including the fixed effects of the Mg source, method, and their interaction. Linear regressions of pH and analyzed soluble Mg contents were performed. Outliers detected were removed (Studentized residuals >3) before fitting the linear regression. For the vinegar test, the best-fit regression was obtained by adding the random effect of Mg sources and time of incubation. A nonlinear relationship between pH and soluble Mg contents was detected for the NH_4NO_3 method, and PROC NLIN was used to fit the data following the exponential function. For experiment 2, the effect of the MgO source, incubation time point, and their interaction on the solubility of Mg from the repeated vinegar test was analyzed. Data on the solubility of MgO in the batch culture experiments were determined for the fixed effect of the MgO source, considering the random effect of the experimental run. Linear regressions between pH readings and soluble Mg contents were performed for experiments 1 and 2. The parameter estimates of linear regression were obtained using the SOLUTION option of the MIXED procedure. The root mean square error (**RMSE**) was calculated according to Robbins et al. (2006), and the concordance correlation coefficient (**CCC**) was calculated using the IML procedure of SAS to accommodate prediction equations.

For the meta-analysis, regression analysis was performed to revise the original equations reported by Schonewille et al. (2008). The first equation was a prediction of true Mg absorption (g/d) from daily intake Mg (g/d) as the only continuous predictor, and the second equation also included dietary K level (g/kg of DM) as a second continuous predictor. The lactation phase (dry and lactation) was included in each regression as an additional discrete predictor. In addition, we tested the model consisting of daily intake of Mg (g/d), dietary K level (low, high), and their interaction. Studies were treated as the random factor in all models. The effect of DMI (kg/d) was also tested in the model but was insignificant ($P > 0.05$), and including DMI as an additional independent factor did not improve the prediction of Mg absorption. This factor was removed from the model. Graphical presentation of the data concerning Mg intake, dietary K level, and true Mg absorption was obtained from the G3D procedure of SAS. Estimations from the original models from Schonewille et al. (2008) were tagged with the word "original," and the revised models in the present meta-analysis were tagged with the word "adjusted." In addition, we evaluated the true Mg absorption (percentage of intake) in response to Mg intake (g/d). The pre-evaluation showed the response was nonlinear. Therefore, we modeled an exponential decay response using the NLMIXED procedure of SAS, with consideration of the study as a random factor.

RESULTS

In Vitro Solubility Method Comparison

We observed similarities across the 3 methods in the ability to dissolve Mg in Mg phosphate and sepiolite (Figure 2). Their pH readings, soluble Mg content, and solubility of Mg remained similar among the 3 methods. Magnesium sulfate had the lowest pH reading compared with the other sources, even though it was highly dissolved in the solution and the solubility was close to 100%. In all methods, MgO samples led to higher pH readings compared with the other sources. The NH_4NO_3 test was superior to the vinegar and ARF tests in dissolving MgO samples leading to double or more soluble Mg contents compared with those found with vinegar and ARF. Consequently, the solubility of MgO sources was 32 to 67% with the NH_4NO_3 test compared with 10 to 17% from the other 2 methods ($P < 0.05$). Despite these differences in the extent of Mg solubility, all methods showed a lower solubility of MgO-D compared with MgO-A and MgO-C, except that the gap was larger with the NH_4NO_3 method.

As in the vinegar method, positive relationships between pH readings and soluble Mg contents were also observed with the NH_4NO_3 and ARF methods (Figure 3). Magnesium sulfate was the outlier and was excluded from the regression analysis. The prediction equation of ARF method was $Y = (36.077 \times pH) - 234.22$ ($P < 0.001$, RMSE = 9.78). Interestingly, the repeatability among replicates was better, and so a better distinction between the Mg sources was obtained with NH_4NO_3

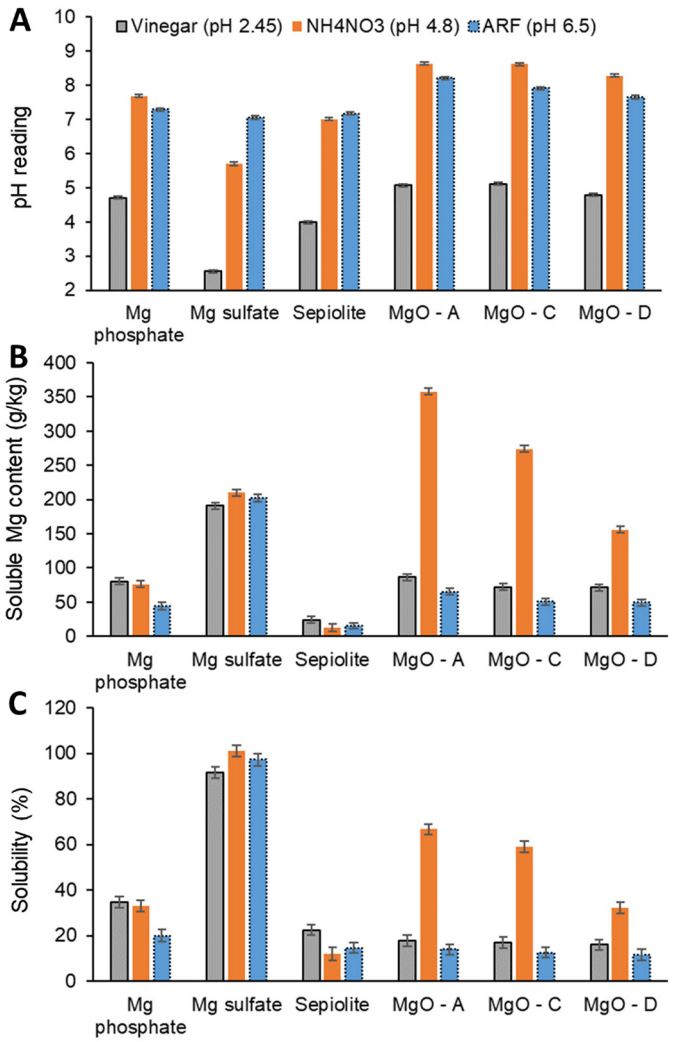

Figure 2. Comparisons of chemical tests using 5% acetic acid solution vol/vol (vinegar), 1 *M* ammonium nitrate solution (NH_4NO_3), and a modified artificial ruminal fluid buffer (ARF) on pH reading, soluble Mg contents (g/kg), and relative solubility (percentage of soluble Mg in total Mg content of original material) of different supplemental Mg sources. MgO = magnesium oxide, Mg sulfate = magnesium sulfate, Mg phosphate = magnesium phosphate. Different MgO letter designations represent sources of magnesium oxide from different geographical origins. Values in brackets in the legend are the initial pH of the respective solution before incubation at 0.5, 24, or 48 h (experiment 1). Error bars represent SE.

than with ARF. However, for NH_4NO_3, the pH range became disproportionate to the soluble Mg content when the soluble content exceeded 250 g/kg, thereby resulting in a nonlinear relationship. The data were then fitted as follows: $Y = 0.00136e^{(1.4244 \times pH)}$ ($P < 0.001$, RMSE = 43.15).

Vinegar Test and Validation

From experiment 1, we chose the vinegar test as the candidate method. First, we focused on its time-dependent performance. Screening of pH development along multiple incubation time points showed that the differences among Mg sources were detected already at 0.5 h, and, as time progressed, the gaps between sources were more apparent, with 3 h of incubation revealing the difference between sepiolite and bentonite (Supplemental Figure S2, https://data.mendeley.com/datasets/9v85ry8t3s/2). An additional test using only MgO sources (MgO-C to -H) at 0.5 and 3 h of incubation also underlined an interaction of source and time ($P < 0.001$; Table 2). We found that MgO-H, which is a premium grade, showed the highest pH reading, whereas MgO-D and MgO-E showed the lowest pH readings compared with the rest of the MgO sources ($P < 0.05$). Increased incubation time increased the pH of the vinegar solution only for MgO-C, -D, and -E ($P < 0.05$) and showed only a tendency for MgO-F ($P = 0.07$), but no difference was observed for MgO-G and -H. As shown in Figure 3A, the regression equation between soluble Mg content and pH reading of the 24-h incubation batch deviated more from those of the shorter incubation times.

In experiment 2, we observed similar results regarding the solubility of MgO in the previous experiment (MgO-A to -D; Figure 4). Specifically, MgO-D was found to have the poorest solubility, and MgO-A was the best source in terms of solubility. Among the 12 new MgO sources, MgO-I, -N, -P, -Q, and -R showed lower solubility ($P < 0.05$), whereas the remaining sources showed high solubility like that of the MgO standard. The sources with poor solubility (<20%) at 0.5 h of incubation showed increased solubility with 3 h of incubation, whereas the highly soluble sources reached solubility of 30 to 35% at 0.5 h of incubation and remained unchanged with time. In general, higher solubility values (15–70%) of MgO sources were obtained from the 24-h batch culture experiments than those observed with the vinegar test. Nevertheless, the changes observed in the vinegar test were in line with the batch culture experiments for most of the sources. Exceptions were for MgO-I, -N, -P, and -Q when comparing them to MgO-D. These samples showed low solubility comparable to MgO-D with the vinegar test, but their solubility was substantially higher in the 24-h batch culture.

Relationships between the pH of the solution and soluble Mg content obtained from experiments 1 and 2 are shown in Figure 5. The majority of the data showed a strong linear positive response, whereas Mg sulfate and Mg acetate, clinoptilolite, and a cluster of MgO were detected as outliers. By excluding these outliers and adjusting variations from Mg sources and time of incubation, similar linear regressions were obtained from both experiments 1 and 2 (Figure 5). A global

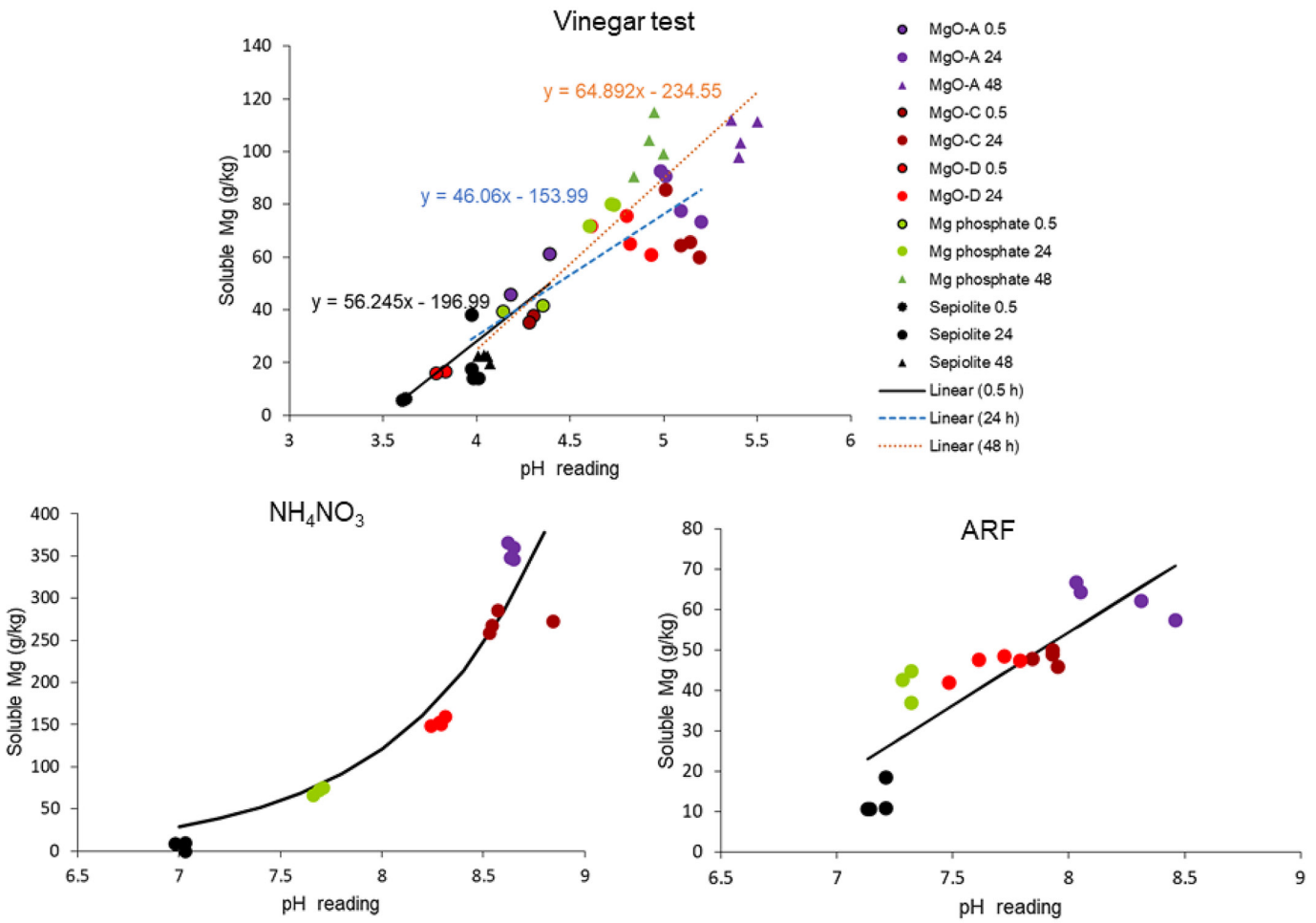

Figure 3. Relationship between analyzed soluble Mg contents (Y, g/kg) and pH readings of the solution (x) after incubation (experiment 1). (A) Vinegar test at 0.5, 24, and 48 h using vinegar solution (5% acetic acid, vol/vol). (B) NH_4NO_3 test for 24-h incubation using 1 *M* ammonium nitrate solution (NH_4NO_3). Y (g/kg) = $0.00136e^{(1.4244 \times pH)}$; $P < 0.001$, root mean square error (RMSE) = 43.15. (C) Artificial ruminal fluid (ARF) test using a modified ARF buffer, Y (g/kg) = (36.077 × pH) − 234.22; $P < 0.001$, RMSE = 9.78. MgO = magnesium oxide, Mg phosphate = magnesium phosphate. Different MgO letter designations represent sources of magnesium oxide from different geographical origins.

linear regression using both experiments led to the prediction equation as follows:

$$\text{Soluble Mg content (g/kg)} = -142.9\ (\pm 14.9) + 44.46\ (\pm 2.55) \times \text{pH};$$

RMSE = 10.2, *P* slope <0.001, and CCC = 0.953.

Meta-Analysis of In Vivo Mg Absorption

The stage of lactation (dry vs. lactation) did not express distinct differences in true Mg absorption (g/d) at a given Mg intake (Figure 6). The differences detected at higher Mg intakes were confounded by the dietary K factor. Moreover, the equations adjusted according to the stage of lactation did not improve the prediction. Therefore, this factor was excluded from any prediction equations in the present study. True Mg absorption (Y, g/d) linearly increased with increasing Mg intake (g/d). The regression equation was Y = −0.037 (±1.03, $P = 0.97$) + (0.2453 × Mg intake, g/d); *P* slope <0.001, and RMSE = 2.34. On a relative scale (percentage of intake), on average 20% of Mg intake was absorbed when the Mg intake was 20 g/d or more. Greater variations among the Mg absorption values (10–40%) were found at higher Mg intake amounts (>60 g/d).

Figure 7A shows the data distribution of the target variables and Figure 7B reveals the interference of dietary K on Mg absorption. We detected a significant interaction between Mg intake and dietary K category ($P < 0.001$). Accordingly, 2 adjusted equations based on the dietary K category were acquired, as follows:

Table 2. pH readings of the solution following the incubation of 6 different magnesium oxide (MgO) sources (experiment 1)[1]

Incubation time	MgO-C	MgO-D	MgO-E	MgO-F	MgO-G	MgO-H	SEM	*P*-value		
								Source	Time	Interaction
0.5 h	4.38[c]	4.07[d]	3.85[d]	4.70[b]	4.62[bc]	9.43[a]	0.05	<0.001	<0.001	<0.001
3 h	4.68[c]*	4.34[d]*	4.38[d]*	4.95[b]	4.78[bc]	9.41[a]				

[a–d]Values in the same row carrying different superscript letters differ significantly according to Tukey's test ($P < 0.05$).
[1]Letter designations indicate magnesium sources differing in geographical origins, manufacturers, and particle sizes.
*Values with asterisks differ significantly ($P < 0.05$) from the respective 0.5-h value according to Tukey's test.

$$\text{True Mg absorption (g/d)} = -1.9273\ (\pm 1.16,\ P = 0.11) + 0.3395\ (\pm 0.025,\ P < 0.001) \times \text{Mg intake (g/d)},\ \text{when dietary K} \leq 20\ \text{g/kg DM};$$

and

$$\text{True Mg absorption (g/d)} = 0.154\ (\pm 1.06,\ P = 0.05) + 0.209\ (\pm 0.026,\ P < 0.001) \times \text{Mg intake (g/d)},\ \text{when dietary K} > 20\ \text{g/kg DM};$$

$$\text{RMSE} = 2.19.$$

We compared the adjusted equations with the original equation of Schonewille et al. (2008): True Mg absorption (g/d) = 3.6 + 0.20 × Mg intake (g/d) − 0.08 × dietary K (g/kg DM); Figure 7B–7F. Our equation from high K resulted in a prediction very close to that of Schonewille et al. (2008; Figure 7B). The additional equation of low K improved the accuracy of the estimation (Figure 7D, 7F) compared with the original model (Figure 7C, 7E). The rho (precision) and CCC of the adjusted model were 0.924 and 0.922, respectively, and those of the original model were 0.888 and 0.845, respectively. Specifically, the original model can underestimate the Mg absorption in the case of high Mg intake (>50 mg/d) but low dietary K levels. However, our adjusted model underestimated the absorption when Mg intake was below 10 g/d. On a relative scale, the adjusted model led to ~20% over- and underestimations of most of the data (Figure 7F), whereas the deviation found with the original model often reached twice as

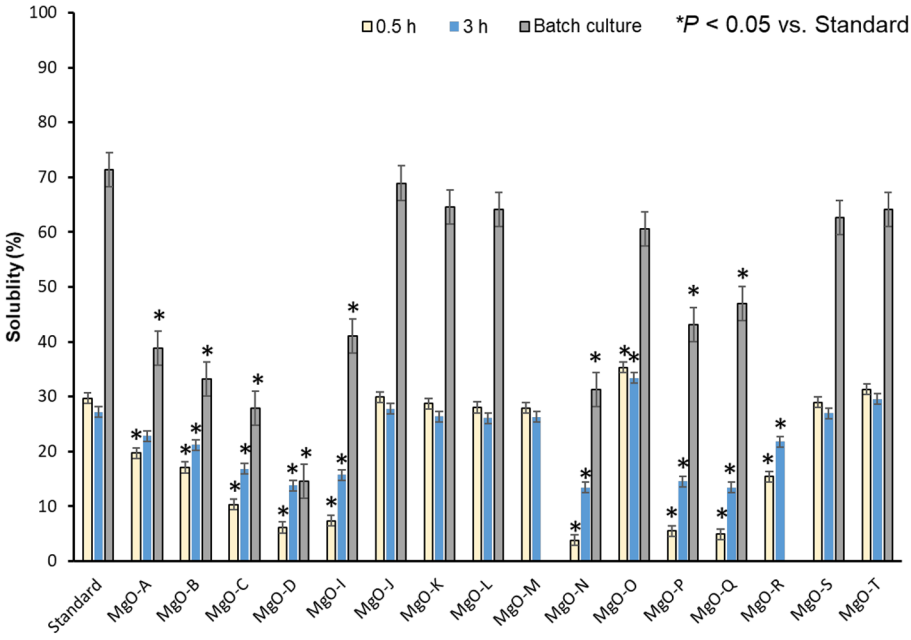

Figure 4. Solubility of Mg of magnesium oxide (MgO) samples subject to the vinegar test for 0.5 h and 3 h of incubation (MgO source $P < 0.001$, time $P < 0.001$, and source × time $P < 0.001$) or 24-h incubation of a batch culture with rumen inoculum (MgO source $P < 0.001$; experiment 2). Different MgO letter designations represent sources of magnesium oxide from different geographical origins. Differences in the solubility between 0.5 vs. 3 h of the vinegar test were detected ($P < 0.05$) for MgO-C, -D, -I, -N, -P, -Q, and -R. Error bars represent SE.

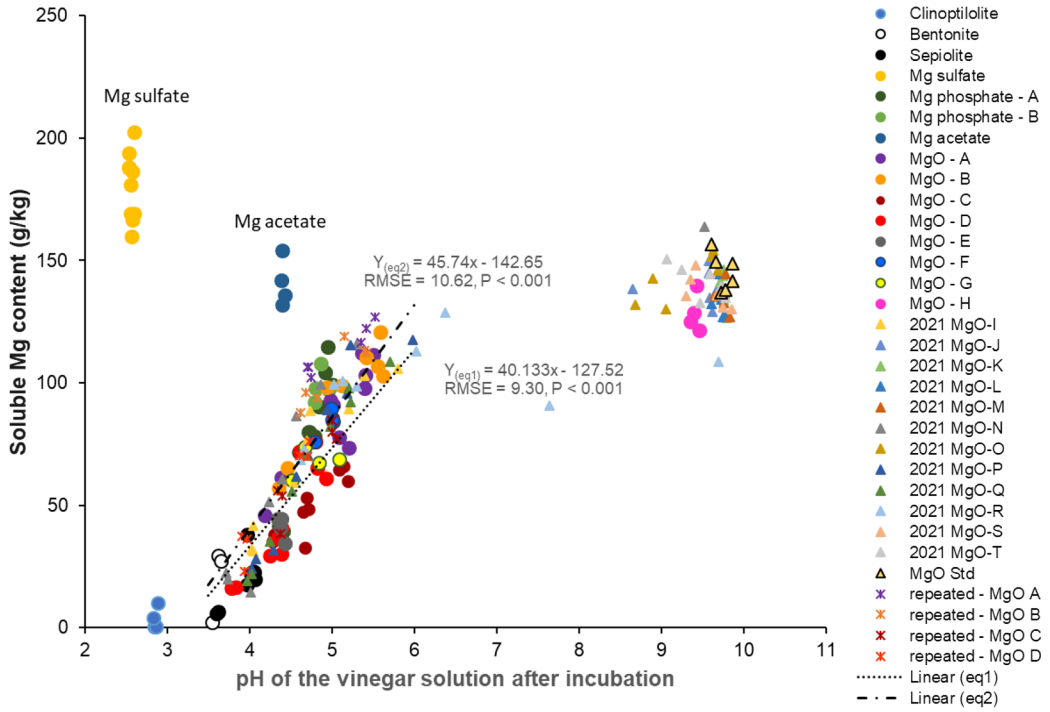

Figure 5. Relationship between pH readings of the solution after incubation with 5% acetic acid solution vol/vol and analyzed soluble Mg contents of different supplemental Mg sources (MgO = magnesium oxide, Mg sulfate = magnesium sulfate, Mg phosphate = magnesium phosphate). Eq1 and Eq2 represent the regression equations of experiments 1 and 2, respectively. A description of the Mg sources used for each experiment is listed in Table 1. RMSE = root mean square error.

high (Figure 7E). Still, for both models, data of the same study tended to be clustered in the overestimation or underestimation area (Table 3).

DISCUSSION

Solubility is a prerequisite to absorption and is an important determining factor for the bioavailability of Mg (Martens and Stumpff, 2019). Based on the solubility and alkaline properties of Mg sources, we demonstrated that chemical tests, especially the vinegar test, could be used for comparisons in the solubility of diverse Mg sources with alkaline properties, except for Mg acetate and Mg sulfate. This was expected, because both of these Mg sources have low Mg contents and Mg sulfate provides no alkaline reaction (Beede, 2017). Magnesium sulfate is also used as anionic salt in close-up cow diets, to help metabolically acidify cows to increase their ability to mobilize bone calcium and thus aid in the prevention of periparturient hypocalcemia (Beede, 2017). Martens and Stumpff (2019) raised some awareness of the practical use of the vinegar test based on the brief concept of the method and small data from the previous research. We showed here that the contents of soluble Mg from supplemental sources could be accurately predicted from the pH values of vinegar solutions regardless of Mg sources and reaction time. Indeed, this shows the practicality of this method in estimating Mg solubility. Such regression equations can be useful for feed mills and for nutritionists without access to Mg analysis. Notably, when using the vinegar test, the reliable prediction is in the pH range of 4 to 6. Simply put, pH values around 4.0 would suggest soluble Mg contents of approximately 40 g/kg of Mg source, and the soluble Mg content doubles for every 1 pH unit. Goff (2014) suggested that the best MgO sources bring the pH up to 8.2 and the worst to 3.8. Similarly, we observed that pH values below 4 suggest poor to no soluble Mg contents. However, a cluster of MgO samples showed pH values of 8 to 10, which were not aligned with the linear regression. The MgO standard also fell into this cluster. The data indicate that these MgO sources had higher alkaline properties at a given soluble Mg content compared with the rest of the Mg sources, which may suggest that these sources are of premium quality and are likely used as antacid components in the grain-rich rations.

In the present work, some other common Mg sources such as Mg carbonate ($MgCO_3$) and calcium Mg carbonate ($CaMg(CO_3)_2$) were not included, and so the

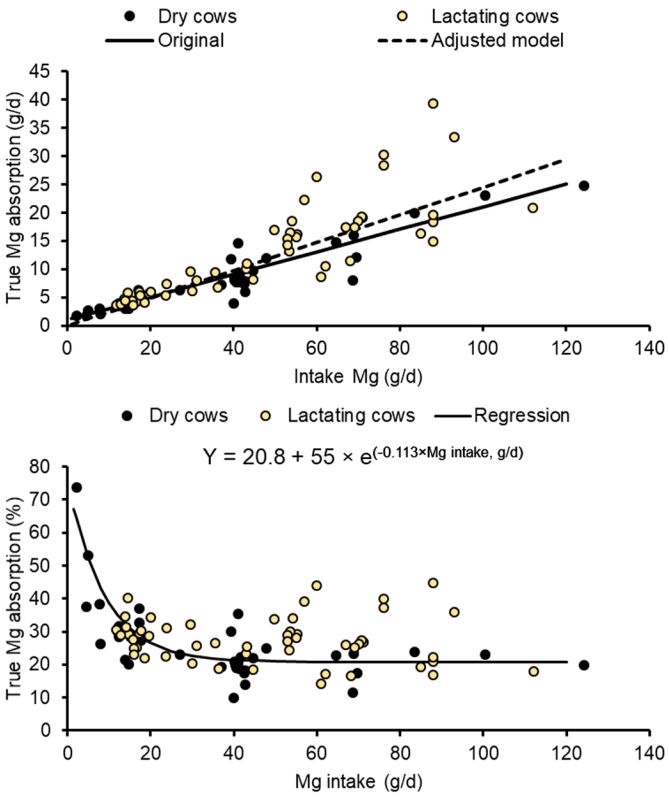

Figure 6. True Mg absorption in response to Mg intake in dairy cows. The upper panel shows the effect on absolute Mg absorption (g/d) and the lower panel the effect on relative absorption (percentage of intake). In the top panel, the regression of the original equation (Schonewille et al., 2008) is Y = 1.3 + 0.20 × Mg intake (g/d) and that of the adjusted model is Y = −0.037 (±1.03, $P = 0.97$) + 0.245 (±0.017, $P < 0.001$) × Mg intake (g/d). Data points distinguish the lactation phase. However, lactation did not have an effect and was not included in the model, and the regression was performed across all data.

applicability of the vinegar test for carbonate sources cannot be ascertained. Carbonate sources have alkaline properties, and so, in theory, the vinegar test should be applicable. These carbonate sources contain lower Mg contents but have a pH-elevating effect equivalent to MgO (Schaefer et al., 1982; Agustinho et al., 2022; Razzaghi et al., 2022), indicating its higher solubility and, for calcium Mg carbonate, the influence of other alkalizing components (calcium carbonate) in the composition. An adjusted protocol and a different prediction equation for soluble Mg contents of the vinegar test might be necessary for sources with interference of other antacid components such as calcium Mg carbonate and marketed products combined with limestone, for example. Further evaluations are needed to confirm this.

Among all the chemical tests performed in the present study, the vinegar test is the most promising method due to its reliability and simplicity. However, it also has some limitations, which may be the general lack of chemical tests to account for the influence of ruminal microbiota and other organic compounds that may influence Mg solubility (Lough et al., 1990). We found that the treatment differences observed in the 24-h batch culture experiments resembled those found with the vinegar test in most cases. However, the solubility values were much lower, with the vinegar test ranging from 5 to 35%, and the values in batch cultures ranging from 30% to as high as 70% (e.g., the MgO standard). The greater values in the batch culture could be partially explained by the substantially longer incubation time. Longer incubation time seems important for slowly soluble sources. For instance, increasing incubation from 0.5 to 3 h significantly increased the solubility of MgO-A, -B, -C, -D, -I, -N, -P, -Q, and -R in the vinegar test. The other sources that reached 30 to 35% solubility within 0.5 h of incubation remained unchanged with time. These samples reached a solubility of almost 70% in 24-h batch culture experiments. That being said, the current vinegar test protocol could accommodate a maximum of 35% soluble contents of Mg from inorganic sources. Because of that, it cannot distinguish good from premium MgO sources, but the distinction would be of minor importance. Of note, the vinegar test could lead to misinterpretation of some sources, compared with the results of the batch culture. For instance, MgO-D was a truly low soluble source that showed very low Mg solubility in all tests and time points. On the contrary, MgO-I, -N, -P, and -Q seemed to be inert sources and so they needed a longer time (>3.0 h) to liberate the Mg. The inert sources did not appear superior to MgO-D in the vinegar test, but they did in the batch culture experiments. It seems that a sufficiently long vinegar test is essential when working with inert sources. Because time efficiency in assessing the quality of raw materials is crucial in feed mills, the 3-h vinegar test would likely be a quick and optimal method for ranking and screening supplemental MgO sources. Without time pressure, increasing incubation time can be recommended to spot inert sources.

The vinegar test can be used to compare supplemental Mg sources and may reflect the differences under rumen conditions. Likewise, in vitro solubility of MgO sources has been shown to be related to in vivo solubility (Schonewille et al., 1992; Tsiplakou et al., 2017). Furthermore, Xin et al. (1989) showed that MgO sources that were less soluble in vitro led to lower ruminal Mg concentrations. However, transferring in vitro solubility values to in vivo values is challenging. Tsiplakou et al. (2017) performed in vivo solubility of MgO using the nylon-bag technique and found that the in vivo solubility was lower than their in vitro solubility test using the ammonium nitrate solution (i.e., the NH_4NO_3 test

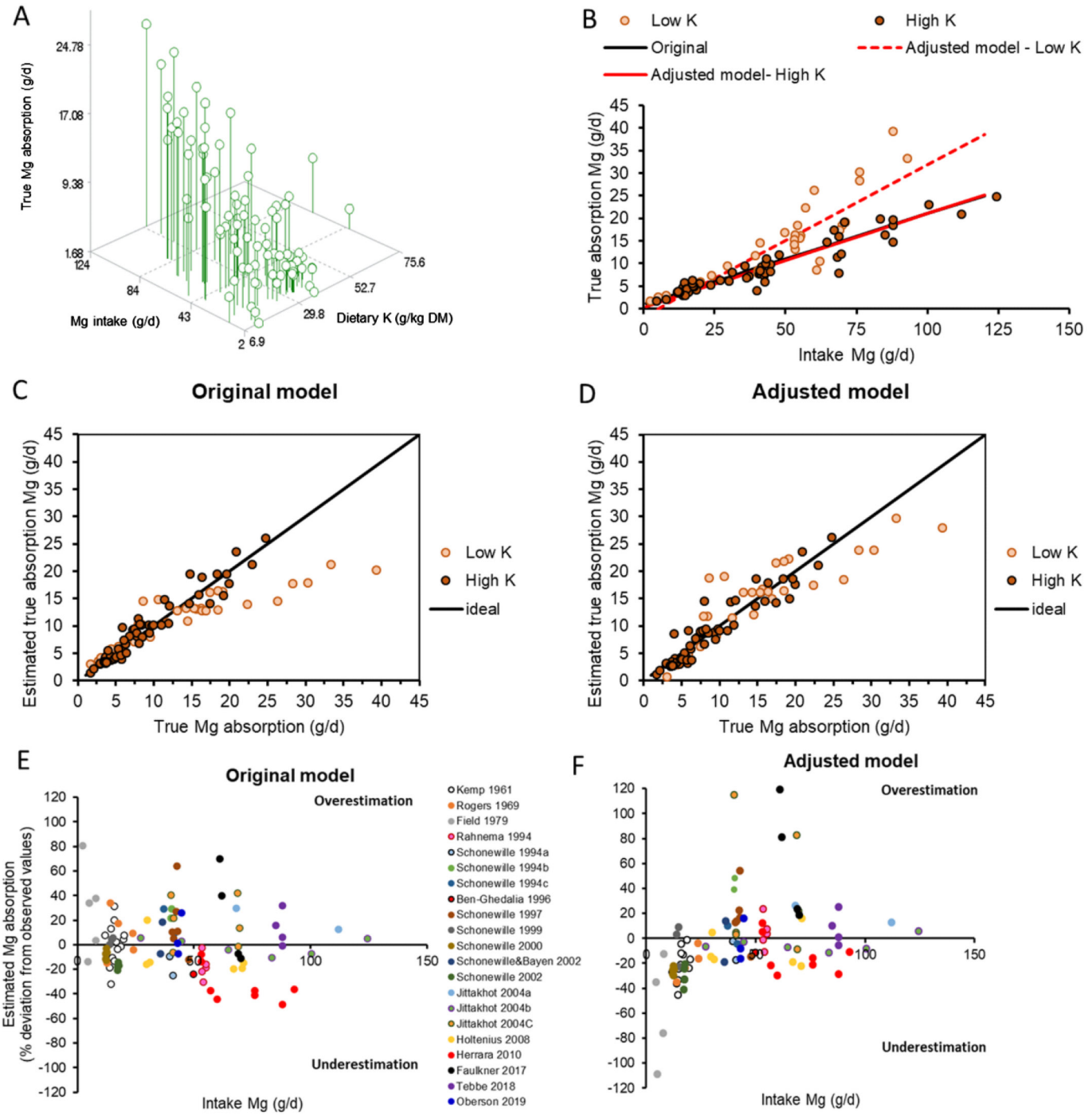

Figure 7. Effect of dietary K level on true Mg absorption. (A) Data point distribution. (B) Linear relationship between true Mg absorption and intake of Mg. (C–F) Deviations of estimations from ideal values. Estimated values were calculated using the original equation, Schonewille et al., 2008: Y = 3.6 + 0.20 × Mg intake (g/d) − 0.08 × dietary K (g/kg DM); or the adjusted models for low (≤20 g/kg DM, Y = −1.9273 [±1.16, $P = 0.11$] + 0.3395 [±0.025, $P < 0.001$] × Mg intake [g/d]) or high dietary K levels (>20 g/kg DM, Y = 0.154 [±1.06, $P = 0.05$] + 0.209 [±0.026, $P < 0.001$] × Mg intake [g/d]). Sources of original model data (chronologically): Kemp et al., 1961; Rogers and van't Klooster, 1969; Field and Suttle, 1979; Rahnema et al., 1994; Schonewille et al., 1994a,b,c; Ben-Ghedalia et al., 1996; Schonewille et al., 1997, 1999, 2000; Schonewille and Bayen, 2002; Schonewille et al., 2002; Jittakhot et al., 2004a,b,c; Holtenius et al., 2008; Herrara et al., 2010; Faulkner et al., 2017; Tebbe et al., 2018; Oberson et al., 2019.

Table 3. Descriptive statistics of the database used for the meta-analysis

Variable	N	Mean	SD	Minimum	Maximum	Median
Nonlactating (dry cows and pregnant heifers)						
BW, kg	41	656	130	350	790	700
Feed intake, kg DM/d	41	6.73	1.04	4.4	8.9	6.8
Dietary Mg, g/kg DM	41	5.09	3.65	0.45	17.34	4.64
Dietary K, g/kg DM	41	32.4	14.2	11.2	75.6	31.2
Intake Mg, g/d	41	35.9	27.01	2.3	124.3	40.0
Intake K, g/d	41	220.1	113.3	55.0	607.0	212.0
Fecal Mg output, g/d	41	30.7	22.18	2.0	102.3	32.4
Apparent absorption, g/d	41	5.3	5.4	0.3	22.0	4.6
Apparent absorption, %	41	13.08	6.61	1.9	30.1	12.7
Endogenous Mg, g/d	41	2.62	0.52	1.4	3.16	2.8
True Mg absorption, g/d	41	7.91	5.54	1.68	24.78	7.18
True Mg absorption, %	41	26.1	11.1	9.9	73.7	23.0
Lactating cows						
BW, kg	53	574	78	440	732	575
Feed intake, kg DM/d	53	16.84	5.09	9.0	26.3	16.9
Dietary Mg, g/kg DM	53	2.59	1.08	1.08	6.25	2.52
Dietary K, g/kg DM	53	22.21	8.71	6.9	41.1	20.0
Intake Mg, g/d	53	46.6	27.1	11.8	112.0	49.8
Intake K, g/d	53	357.2	130.6	62.0	625.3	339
Fecal Mg output, g/d	53	36.22	20.73	10.2	93.5	36.0
Apparent absorption, g/d	53	10.41	8.30	1.6	37.0	8.0
Apparent absorption, %	53	20.68	7.66	9.8	42.0	19.4
Endogenous Mg, g/d	53	2.30	0.31	1.76	2.9	2.3
True Mg absorption, g/d	53	12.72	8.43	3.6	39.3	10.56
True Mg absorption, %	53	27.68	6.99	14.1	44.66	27.2

in the present study), which we also showed to result in higher solubility of MgO than the other 2 methods. In general, we observed low solubility values of the Mg sources (up to 35%) in the vinegar test. In line with our findings, relatively low in vitro solubility of MgO has been reported in other studies using acid solutions (Xin et al., 1989; Lindberg et al., 1990), simulation of the rumen (Beede et al., 1992), or simulation of gastric digestion (Blancquaert et al., 2019). Xin et al. (1989) compared 3 MgO sources. They showed that the solubility in an acidic solution of MgO sources could be as low as <5% in the source with low reactivity, compared with 22.8% for the source with the highest reactivity. Simulation of the abomasal system showed greater solubility of MgO of up to 50% (Beede et al., 1992). We questioned whether these low solubility values of MgO can in some way logically match or explain both solubility and absorption values of Mg observed in vivo. Because not enough studies have tested ruminal solubility, we resourced studies testing Mg absorption. Similar to the low in vitro solubility of MgO reported in the literature, low digestibility of Mg has been described in dairy cows (Martens and Stumpff, 2019), and digestibility in these studies was measured as apparent absorption (intake − fecal output). Thus, the digestibility can be confounded by the endogenous loss of Mg, which may be linked to the discrepancies between studies in dry and lactation cows (e.g., Jittakhot et al., 2004a,c; Holtenius et al., 2008). When balancing out these factors, the current meta-analysis revealed a true Mg absorption of about 20% of the intake similarly in nonproducing cows and cows in lactation. These values were not too far apart from the in vitro solubility of MgO (5–35%) detected with the vinegar test in the present study, which may suggest that generally low solubility of MgO contributes, to some extent, to low Mg absorption in dairy cattle. Although most studies included in the current meta-analysis used MgO, they differed in various factors that can influence the fate of Mg in the rumen, which is discussed subsequently.

Magnesium in diets originates from both organic and inorganic sources, and some factors also affect ruminal absorption of solubilized Mg. Dietary K is a major competing factor for ruminal Mg absorption (Martens and Stumpff, 2019). Results of our meta-analysis also supported this, and we showed a strategy to improve the estimation based on dietary K level. The equation previously established by Schonewille et al. (2008) was derived from many studies using byproduct-based concentrates, which resulted in a concomitantly high K supply. So, our new prediction for the high K group (>20 g/kg DM) aligned perfectly with that of Schonewille et al. (2008). The updated database revealed that a different prediction was necessary when high-Mg but low-K diets are used. Still, with this improvement in the prediction, unaccountable factors may lead to over- or underestimations inherent to individual studies. We ruled out the stage of lactation, although this

must be interpreted with caution because we did not have data on dry cows fed high-Mg but low-K diets to ascertain the dominant role of this dietary factor over the lactation stage. Nevertheless, we standardized the dietary K and Mg intake as a result of different intake levels, different feed ingredients, and variation in mineral supplementation among the studies. Notably, most studies used MgO as the supplemental source of Mg. However, likely, the MgO sources differed greatly in quality, as the geographical origin and the calcination temperature of ores affect the availability of inorganic sources (Beede, 2017). Unfortunately, most studies did not report information on supplemental sources. Given that Mg sources with higher solubility will increase ruminal Mg concentrations (Xin et al., 1989) and thus will increase absorption, chemical methods can provide a better understanding of the potentially available Mg contents in different sources. At the current stage, the use of in vitro solubility tests could improve the precision of feed formulation. The current gap of knowledge regarding the quality of supplemental Mg sources on the absorption reinforces the necessity of using the vinegar test as an additional method in Mg nutrition research to acquire enough data that could be integrated and improve the prediction equations. This approach will aid success in increasing the efficient use of raw materials and lowering the burden on the environment from the excretion of unavailable or oversupplied minerals. Several other variables such as the particle size and source (feed-borne vs. inorganic) of Mg, ruminal pH, and passage rate affect Mg solubility as well as the residence time of Mg particles in the rumen. These factors may also further contribute to unexplained variations in Mg absorption among studies, albeit the overall contribution might be small. Only a handful of the studies in the current database reported mean ruminal pH, which consistently was >6.5 and thus within a range for low Mg solubility (Dalley et al., 1997). It would require acidosis conditions to modify Mg solubility drastically. Oberson et al. (2019) emphasized the dependency of Mg absorption on the rumen volume but not the passage rate. Furthermore, the fate of heavy and fine particles in the gastrointestinal tract might not follow that of feed particles. A study using a sand-contaminated diet in dairy cattle showed that these heavy particles reside mainly in the ventral rumen and have a high degree of washout from the rumen (84% ± 14) and a 2-d residence time in the gastrointestinal tract (recovered in feces). The passage rate might be more critical for the release of feed-borne Mg than for inorganic sources.

CONCLUSIONS

The present study supports the concept of using simple chemical tests to differentiate Mg supplemental sources with different qualities. Among the methods investigated, the vinegar test proved to be the most promising method for screening and ranking Mg sources with alkaline properties. We provided an equation to accurately predict soluble Mg contents from pH readings when using the vinegar test. The equation could benefit nutritionists and feed mills without access to Mg analysis. The in vitro solubility values of MgO (5–35%) in the vinegar test fell within the range of true Mg absorption in vivo (10–40%), as revealed by the performed meta-analysis. At this stage, the vinegar test can assist in the selection of better (more soluble) Mg sources and can provide a correction factor for Mg sources to improve the precision of feed formulation. In the meta-analysis, we refined the prediction equation for true Mg absorption based on dietary K level.

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