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ReaShure®

Precision Release Choline

Balchem Research Summary

A comparison of two commercially available rumen-protected choline products (ReaShure; Balchem Inc., Montvale, NJ and CholiGem; Kemin Industries, Inc., Des Moines, IA) using several *in vitro / in situ* methods to determine feed stability and rumen protection levels

A summary of 4 studies conducted by Clay Zimmerman, Ph.D. and Kari Estes, M.S.,current employees of Balchem Corporation.

Introduction

Measuring the bioavailability of a rumen-protected nutrient is critical to assessing its value to the animal and to the dairy enterprise. Scientists have successfully developed validated *in vivo* methodologies for assessing the bioavailability of other rumen-protected nutrients like methionine (using a milk selenomethionine technique; Weiss and St-Pierre, 2009) and lysine (using stable isotope methods; Estes et al., 2018 and Rubelo and Lee, 2024). However, we currently lack a recognized *in vivo* procedure specifically designed to measure choline bioavailability in dairy cows. Unlike other nutrients that possess well-established biomarkers reflecting intake or status, choline lacks universally accepted biomarkers that directly reflect its intake or status in the body, complicating its bioavailability assessment.

Several *in vitro* methodologies have been developed in an attempt to evaluate choline bioavailability, but they've been proven inadequate in providing accurate assessments. Nonetheless, these methods still offer useful and relatively precise insights into some factors influencing nutrient absorption.

Three primary factors significantly affect the overall bioavailability of rumen protected nutrients: feed stability, rumen protection and intestinal absorption. *In vitro* models have demonstrated effectiveness in accurately measuring feed stability and rumen protection but have notably struggled in predicting intestinal absorption.

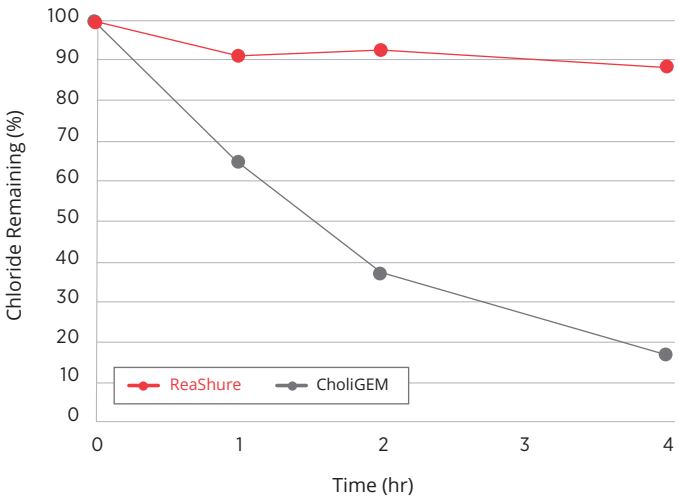
In this paper we'll evaluate a series of studies that compare two commercially available rumen-protected choline products (ReaShure® [28.8% choline chloride; Balchem® Inc., Montvale, NJ] and CholiGem™ [60% choline chloride; Kemin® Industries, Inc., Des Moines, IA; Figure 1]) using several *in vitro/in situ* methods to determine feed stability and rumen protection levels. Unless otherwise noted, each of these studies was funded and conducted by Balchem or Balchem affiliates.

Methods and Results

Trial 1: *In Vitro* Rumen Stability Assay - Water Medium The water test is a relatively quick and easy procedure to illustrate the stability of RP-choline products. In this procedure, one sample of each product (two grams) is individually incubated in 100 ml of room temperature, distilled water for 1, 2 and 4 hr. Chloride content (proxy for choline) of the water is measured at each incubation timepoint. Any chloride released in the solution at each timepoint is considered to be the unstable fraction of each product.

The results from the water test are shown in Figure 1. While this technique uses water and not rumen fluid, it could be assumed that any inferences made regarding rumen stability from this data set would be, at worst case, over-estimations. With that in mind, after just 1 hour of incubation in room temperature water, roughly 65% of the choline content of CholiGem was remaining. By 4 hrs of incubation, only 17% of the original choline content was considered stable. In contrast, ReaShure remained very stable in the water over time with roughly 88% of the choline remaining after 4 hrs of incubation.

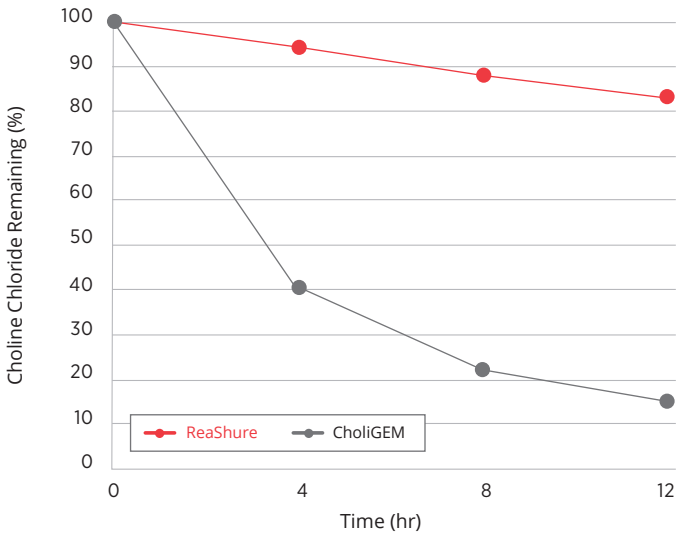
Figure 1 Water Stability of ReaShure and CholiGEM



Trial 2: *In Vitro* Rumen Stability Assay – Buffered Lipase Solution Balchem also evaluated each product for rumen stability using an internally developed *in vitro* procedure that consisted of a buffered solution with lipase enzymes that mimics the rumen environment. Duplicate samples of each product were individually sealed into dacron bags and suspended in the buffer solution maintained at 39°C (102.2°F) under constant agitation (160 rpm mixing rate) for 4, 8, and 12 hr. Aliquots of the solution at each timepoint were collected and analyzed for choline chloride content via a biochemical analyzer (YSI Incorporated, Yellow Springs, OH). Choline measured in the solution was considered to be the rumen degradable fraction of the product.

Figure 2 reports the percent of choline chloride remaining for ReaShure and CholiGem when tested *in vitro* for rumen stability using Balchem's buffered lipase solution. In this methodology, CholiGem lost a majority of its choline after 4 hrs of incubation in the solution. As rumen *in vitro* incubation time increased, CholiGem continued to degrade and by 12 hrs, only 15% of the choline content was remaining. On the contrary, ReaShure was consistently more stable over time and by 12 hrs, 83% of the choline content survived degradation.

Figure 2 ***In vitro*** Rumen Stability of ReaShure and CholiGEM



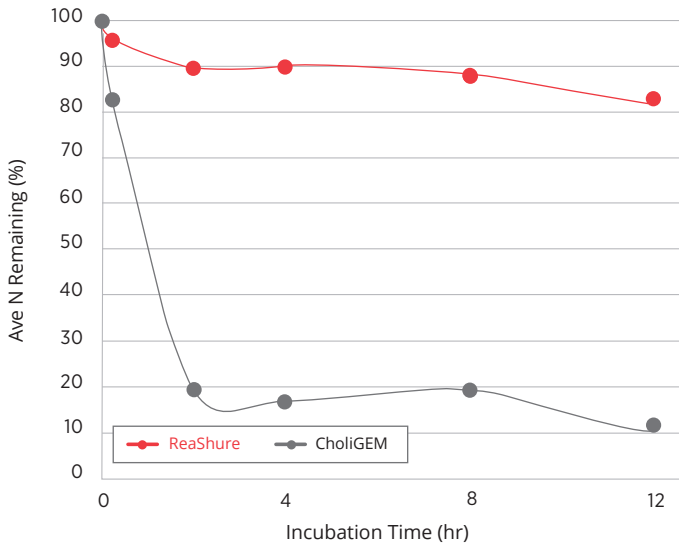
Trial 3*: *In Situ* Rumen Stability Assay Both ReaShure and CholiGem were evaluated at a third-party commercial laboratory (Cumberland Valley Analytical Services [Waynesboro, PA]) for rumen stability using an *in situ* procedure. For the *in situ* analysis, duplicate samples of both products were individually sealed into dacron bags (50 µm pore size, Ankom Technology) and were suspended in the rumen of three cannulated lactating cows for 0.25, 2, 4, 8 and 12 hr. Nitrogen (N; proxy for choline) and dry matter (DM) content were determine for each product at each timepoint. Samples of each product that were not rumen incubated (0 hr) were also analyzed for N and DM content.

Figure 3 reports the percent of choline remaining for ReaShure and CholiGem when tested *in situ* for rumen stability. In this methodology, CholiGem lost roughly 80% of its choline after 8 hrs in the rumen (the typical retention time in the rumen for these types of products). As rumen incubation time increased, CholiGem continued to degrade and by 12 hrs, only 10% of the choline content remained. On the contrary, ReaShure was consistently more stable over time and by 8 hrs, roughly 88% of the choline content survived degradation.

Trial 4*: Total Mixed Ration (TMR) Stability Study Both ReaShure and CholiGem were also evaluated for TMR stability using a modified version of the methods developed by Ji et al (2016) and Ishimaru et al (2019). In this experiment, a corn silage and haylage-based TMR (DM = 38.7%; pH =4.8) was utilized. For each treatment, the TMR (200 g) and the equivalent of 1 g of CC from each product were weighed in triplicate and gently mixed for 30 seconds in a sealable Ziplock freezer bag. Each treatment was then stored unsealed for 0, 6, 12 and 24 hr at 22°C (72°F). At each timepoint, the contents of each Ziplock bag were transferred to a strainer bag (250 µM pore size) and were shaken for 1 min with 1 L of distilled water to facilitate solubilization of any free CC released from the products. A sample of the solution was then taken, filtered (0.45µM disk filter) and analyzed for CC content. Any CC measured in the solution was considered to be the damaged/unstable fraction of the product. Data were analyzed using the MIXED procedure of SAS with the fixed effects of treatment, time and their interaction, and the random effect of bag. Means were separated using the Tukey adjustment.

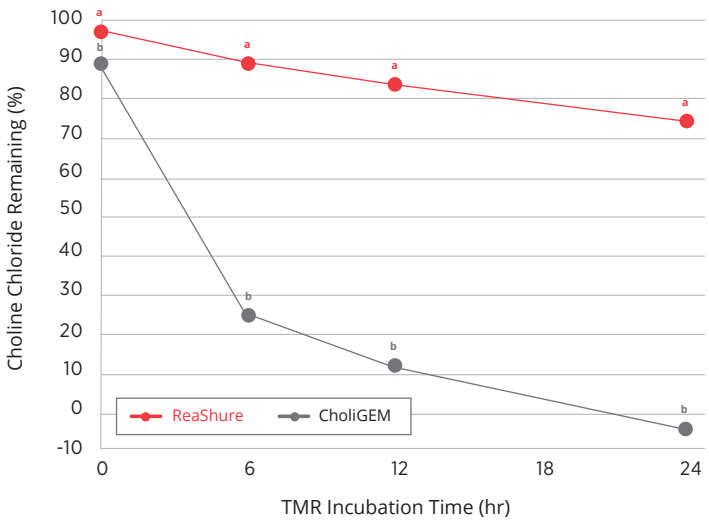
The TMR stability of ReaShure and CholiGem are shown in Figure 4. At all timepoints, CholiGem experienced significant TMR damage compared to ReaShure. After just 6 hrs in the TMR, only 25% of the choline chloride content of CholiGem was remaining. As exposure time increased, CholiGem continued to degrade until none of the product remained after 24 hrs of TMR incubation. While ReaShure did experience damage from the TMR, it was significantly more stable than CholiGem with 75% remaining after sitting in the TMR for 24 hrs.

Figure 3 ***In situ*** Rumen Stability of ReaShure and CholiGEM



Study was conducted at a third party lab (Cumberland Valley Analytical Services [Waynesboro, PA]).

Figure 4 TMR Stability of ReaShure and CholiGEM



[ab]Values with differing superscripts within a timepoint are considered statistically different (P<0.05).
Estes et al. 2024

Conclusions

In this research, CholiGem was unable to adequately withstand either the simulated *(in vitro)* or true *(in situ)* rumen environment with approximately 20% of the choline surviving at 8 hrs. Additionally, CholiGem experienced significant damage from sitting in a TMR over a 24 hr period. ReaShure's encapsulation technology was more effective in protecting choline from water, the rumen and TMR environments when compared to CholiGem.

While these *in vitro* and *in situ* models have demonstrated effectiveness in accurately measuring feed stability and rumen protection, they cannot tell us the true bioavailability of these products. However, ReaShure is backed by 41 peer-reviewed published papers showing positive benefits in transition cow health, calf health and growth as well as milk and colostrum yields. Only one *in vivo* research trial has been published in a peer-reviewed journal with CholiGem.

The *in vitro*, *in situ* and TMR stability data clearly illustrates that ReaShure has improved feed stability and rumen protection when compared to CholiGem.

*These trials were conducted at third-party laboratories.

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