Determining the effect of protozoal inhibitors on protozoal motility to improve ruminant feed efficiency and reduce enteric methane

Honors Research Thesis

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ABSTRACT

Enteric methane production is estimated to contribute 17% of global methane, produced exclusively by a group of archaea known as methanogens, which tend to associate with protozoa as a symbiotic source of substrate (formate or CO_2 and H_2). Recent studies have focused on direct inhibition of methanogens or decreasing the availability of H_2 or formate. Monensin (MON), an antibiotic not used in human therapy, is a feed additive used to improve production efficiency in cattle. Essential oils are non-antibiotic alternatives that selectively inhibit groups of microbes and include products such as Cinnagar® (CIN), a combination of cinnamon and garlic essential oils. This study used 240 videos during an in vitro trial to examine the effects of MON and CIN treatment on protozoal motility. Previous research on these additives decreased an indirect measurement of protozoal volume but had variable consequences for N/cell ratio. We hypothesized that MON and CIN would have additive inhibitory effects on protozoal function and motility. Protozoa were given a control treatment (CON) or treatment with MON, CIN, or MON+CIN in a 2 x 2 factorial arrangement of treatments. Replicate tubes at 3 h post-feeding were analyzed for distance, average speed, and average protozoal area with ImageJ software and using hour 0 as a covariate. The main effect of MON decreased ($P<0.05$) protozoal distance by 134 μm and average speed by 66.2 μm/s. The main effect of CIN decreased ($P<0.05$) average area by 256 μm^2. MON interacted ($P<0.05$) with CIN for average speed, demonstrating CIN decreased MON's inhibition; simple treatment means for CON, MON, CIN, and MON+CIN were 243, 138, 211, and 183 μm/s, respectively. Compared with more tedious and time-consuming protozoal motility methods, the current method will improve efficiency, accuracy, and/or precision for future studies assessing the role of protozoal ecology on enteric methane production in cattle.

INTRODUCTION

Ruminants are unique in their ability to digest fibrous feedstuffs for energy. The ruminant digestive tract contains anaerobic microorganisms bacteria, protozoa, fungi, and archaea, which are capable of degrading starch, cellulose, hemicellulose, and pectin to sugars followed by fermentation. A symbiotic relationship occurs in which the ruminant host maintains this population of microorganisms and, in exchange, utilizes the products, primarily volatile fatty acids (VFAs), as a principal source of energy. Methanogens are in the domain archaea. They convert carbon dioxide (CO_2) and dihydrogen (H_2) to methane (CH_4). Many methanogens associate directly with protozoa by attaching to their surface and utilizing the H_2 or formate (which is converted to CO_2 and H_2 by archaea possessing formate dehydrogenase) produced by protozoa during fermentation.

Ionophores are antimicrobial compounds that are not used in human medicine but are supplied in livestock feed at sub-therapeutic rates to improve efficiency by altering the rumen microbiome. Monensin is an ionophore that inhibits H_2 production by bacteria (especially gram-positives) and thereby inhibits methanogens by the decreased H_2 availability. The role of protozoa in ruminant nutrition has become the topic of much debate and study in recent years (Morgavi et al., 2011). Inhibiting protozoa may have benefits both in decreasing substrates for methane production plus increasing rumen efficiency by eliminating their predation on bacteria (Newbold et al., 2015). It has been found that a linear relationship exists between protozoal concentration and methane production (Newbold et al., 2015). Inhibiting protozoa in order to decrease methane production has been the goal of many studies, but it must be done without decreasing nutrient digestibility and the overall production of the animal. Understanding the precise way in which protozoal inhibitors impact protozoa is necessary for the further development of products that may

decrease enteric methane emission while conserving efficiency. This study provides insight into whether these two particular inhibitors affect protozoal motility and in what way, by looking at distance, direction change, average area, and average speed. Perhaps more importantly, this study contributes directly to developing an efficient method for obtaining motility data in future trials with inhibitors.

Monensin is used in both beef and dairy production throughout North America. It has been banned in the European Union due to recent concerns of feed-grade antibiotics conferring antimicrobial resistance, although no data on monensin have been shown to support this view (Hristov et al., 2013). Monensin generally improves feed efficiency and decreases methane production as a result of that improved efficiency; however, the direct effects on methanogenesis per se are inconsistent (Hristov et al., 2013). Rumen protozoa adapt to monensin, thereby lessening its effects over time and with repeated use (Sylvester et al., 2009). Further research on monensin's specific mode of action is necessary to determine the effect on protozoa and in the future development of products designed to decrease methane production.

Essential oils have decreased methane production in the same way as monensin. Their antimicrobial properties also inhibit gram-positive bacteria, effectively decreasing the amount of H_2 available to methanogens (Hook et al., 2010). The variety of microorganisms present in the rumen have differing susceptibility to different kinds of essential oils, opening the possibility for targeted selection of specific microorganisms (Benchaar & Greathead, 2011). More study is needed to determine if a particular essential oil could be used for the targeted reduction of methane production; understanding how protozoa are impacted by essential oils contributes to this development.

PROBLEM IDENTIFICATION AND JUSTIFICATION

Previous research indicates that monensin and Cinnagar® do not inhibit protozoa by decreasing the number of protozoa present. Instead, research by Ye (2013) indicated that the nitrogen per cell ratio is decreased. Further research by Wagner (unpublished) explored why these results occurred, with the hypothesis that the volume of cells or protein concentration was being decreased. The results of that study were inconclusive, but videography taken during the trial may provide further insight as to the effects of monensin (MON), Cinnagar® (CIN), and their combination (MON+CIN), on rumen protozoa compared with the control (CON). This study aims to develop a method for analyzing video data using ImageJ software, contributing to the understanding of how protozoal motility is affected by the treatments, as well as further the ability to use videography for future protozoal motility study (Schneider et al., 2012).

HYPOTHESIS AND OBJECTIVES

We hypothesized that protozoal motility would be inhibited by MON and CIN, and MON+CIN would contribute an additive inhibition, meaning the combination of the two inhibitors would inhibit protozoa greater than the sum of the main effects of treatments. This hypothesis was tested by individually running each video through ImageJ using the "Tetratracker" plugin, and then compiling the data to determine effects on distance, direction change, average area, and average speed (Schneider et al., 2012).

Objective 1: Develop a method for processing each video, including parameters that contribute to eliminating non-cellular debris.

Objective 2: Utilize the generated data from ImageJ to assess the effects of CON, MON, CIN, and MON+CIN on protozoal motility.

MATERIALS AND METHODS

Objective 1: Develop a method for processing each video, including parameters that contribute to eliminating non-cellular debris.

In the original trial by Wagner (unpublished), protozoa were given a control treatment (CON) or treatment with MON, CIN, or MON+CIN in a 2 x 2 factorial arrangement of treatments with 2 replications. The 4 treatments included 1) CON (feed only), 2) feed + .0043% DM CIN, 3) feed + 2.82 μM MON, and 4) feed + CIN + MON (in the same concentrations). The feed mixture consisted of 70% cellulose, 25% potato starch, and 5% glucose. Treatment concentration was consistent with manufacturer feeding recommendations and was the same as concentrations used in the study conducted by Ye (2013). Nine 10-sec videos were taken using a Nikon D5000 camera of a 0.05 mL sample from each tube at hours 0, 3, and 6. This generated 216 videos for each replication, with a total of 432 videos for the 2 replications that were conducted. A subsample of these videos was taken by processing 5 of the 9 videos from each tube, with the odd numbered videos being chosen for analysis. This left a total of 240 videos for processing. Each video was then converted using VirtualDub software to .AVI format in order to decrease file size by removing audio (VirtualDub). These converted videos were individually processed using ImageJ software (Schneider et al., 2012). Upon opening a single video in ImageJ, they were limited to frames 0 to a maximum of 300. This eliminated the end of videos that were excessively long due to error in recording time and helped shorten the amount of time spent uploading. The videos were then converted to grayscale and adjusted for threshold, which used red density to convert the video to a black and white binary view. Adjustments were made individually for each video, with a goal of setting the threshold at a point where all protozoal cell outlines were highlighted and complete, while taking care to keep as much of the background

and non-protozoal material from being highlighted as possible. After this conversion, a background stack was implemented called "Z-stack". Running Z-stack created an image of what did not change over the course of the 300 frames included in an individual video. These images were essentially subtracted from the video using the image calculator. At this point, the video was ready to be run through Tetratracker, a plugin provided by T. Hennessey from the University of Buffalo that was available within ImageJ. Within Tetratracker, parameters were chosen that best resulted in the program detecting the majority of live protozoa within the video. These parameters were chosen by individually measuring cells in a random selection of videos and repeatedly processing videos with varying parameters to gauge which best allowed for maximal cell detection. Having minimum object area too small resulted in detection of too many feed particles, and maximum object area needed to be large enough to include the larger isotrichid protozoa while limiting the detection of large clumps of feed particles. Setting a maximum velocity and maximum area change decreased the amount the track would jump from one particle to another in subsequent frames. The minimum track length assured only cells that stayed in the video for at least 2 seconds were tracked. The parameters listed below were then used to process all 240 videos and are as follows:

Table 1: Parameters entered into Tetratracker for maximal protozoal identification.

Minimum Object Area ($pixels^2$)	600
Maximum Object Area ($pixels^2$)	55,000
Maximum Velocity (pixels/frame)	400
Maximum Area Change (%)	100
Minimum Track Length (frames)	60
Threshold for turn	5

The program used these parameters to identify tracks of moving objects within the video. Each video with the labeled tracks was observed for accuracy of cell detection. Exceptionally poor quality videos were not used for further analysis. The subsequent data file that was outputted from ImageJ for each video included each track's individual length, distance, direction change, number of frames, first frame, time, maximum speed, average protozoal area, standard area, average perimeter, standard perimeter, average speed, and body lengths per second.

In order to better assess the advantages and disadvantages of using this method of video analysis for studying protozoal motility, the videos were also processed through a subjective motility scoring method that had been used in previous studies (Morris, unpublished). Each video was visually reviewed in its entirety and then given an individual score from 0 to 5, with 0 being no motility and 5 being heightened motility.

Objective 2: Utilize the generated data from ImageJ to assess the effects of MON, CIN, and MON+CIN relative to CON on protozoal motility.

In order to eliminate some tracks that were not protozoa, video data results were compared to the videos with labeled tracks. A majority of tracks with a direction change <0.2 were not protozoa; instead, they were either feed particles or tracks that frequently "skipped" from one particle to another. These individual tracks were then deleted. The lists of tracks from each of the 5 videos per treatment tube were then averaged, and units containing pixels were converted to micrometers. Distance (length), direction change, average protozoal area, and average speed were chosen as the most relevant characteristics from which to determine protozoal motility and treatment effects. Data were analyzed using the Mixed procedure of SAS (SAS Institute, 2014) in a randomized complete block design: fixed effects of treatment, the random blocking effect of inoculation, and a covariate adjusted for dependent variable mean within treatment × replicate

inoculation at 0 h. Treatment sums of squares were partitioned into the main effects and interaction of MON and CIN. Significance was declared at $P \leq 0.05$, and trends were $0.05 < P \leq 0.10$.

RESULTS

Data at 0 h post-feeding showed no statistical differences across treatments. This result was expected and demonstrated uniformity of motility at the onset of treatment. Uniformity at 0 h post-feeding allowed significant differences at 3 h and 6 h post-feeding to be contributed solely to treatment effects. At 3 h post-feeding, MON and MON+CIN both showed significant differences in distance and average speed. The main effect of MON at 3 h post-feeding decreased ($P<0.05$) protozoal distance traveled by 134 μm compared with the diets without MON (Figure 1). The main effect of CIN at 3 h post-feeding decreased ($P<0.05$) average protozoal area by 256 μm^2 compared with the diets without CIN (Figure 2). There was a treatment interaction ($P<0.05$) at 3 h post-feeding in which CIN decreased MON's inhibition of average speed (Figure 3); simple treatment means for CON, MON, CIN, and MON+CIN were 243, 138, 211, and 183 μm/s, respectively.

Figure 1: Distance using ImageJ at 3 h post-feeding with significant differences for the main effect of MON (P=0.04) and MON+CIN (P<0.01).

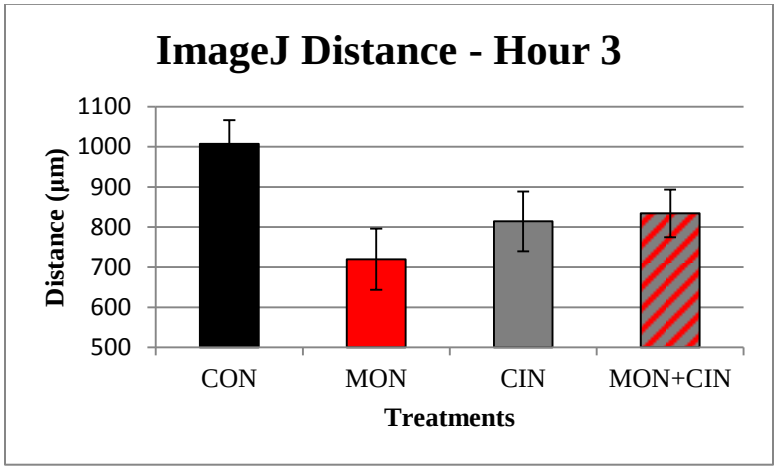


Figure 2: Average area using ImageJ at 3 h post-feeding with significant difference for the main effect of CIN (P=0.04).

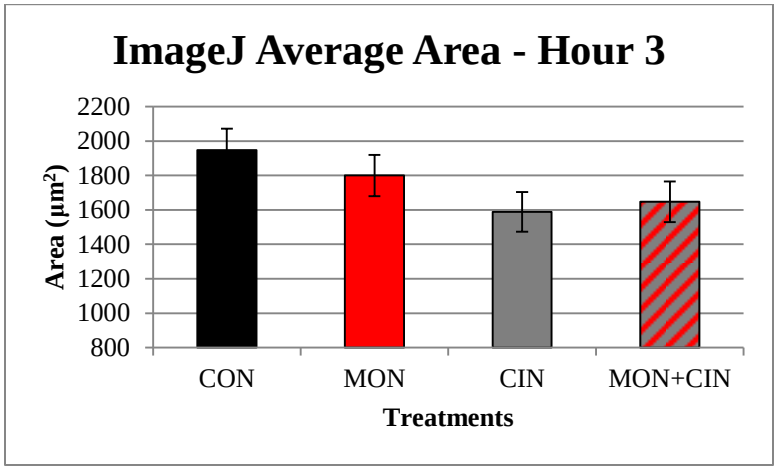
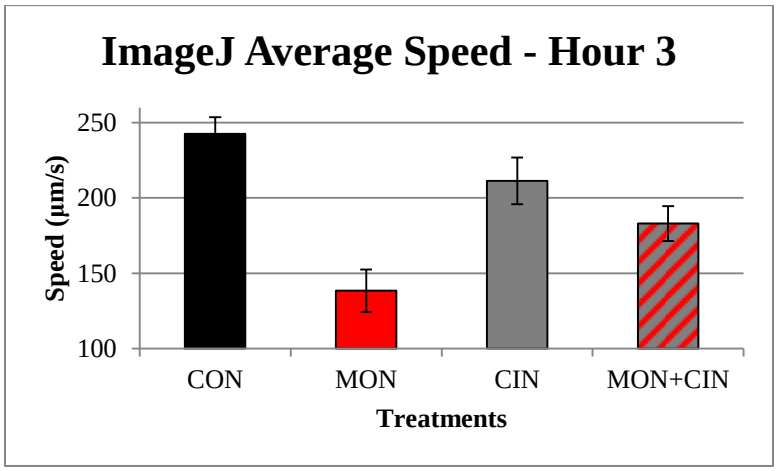


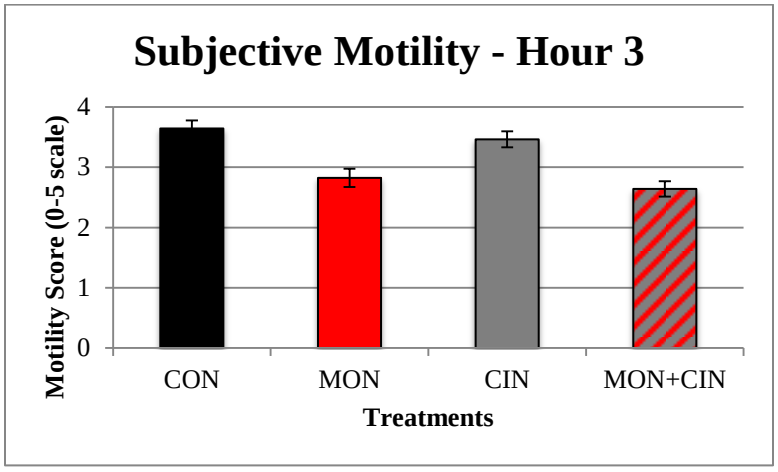
Figure 3: Average speed using ImageJ at 3 h post-feeding with a treatment interaction (P<0.01) of CIN with MON.



No statistical effects were observed for direction change (P>0.15). By 6 h post-feeding, significant differences were noted only for the main effect of MON (P<0.01) on average speed (data not shown).

When these videos were analyzed using subjective motility scoring, the main effect of MON decreased (P<0.05) motility at 3 h and 6 h post-feeding. At 3 h post-feeding, the main effect of MON decreased the motility score by 0.8 (Figure 4).

Figure 4: Subjective motility at 3 h post-feeding with significant difference for MON (P<0.01).



DISCUSSION OF RESULTS

Both methods of video analysis indicated that MON inhibited protozoal motility. ImageJ analysis showed this inhibition to be in the areas of distance and average speed, corresponding with initial inhibitory responses of MON found in previous studies (Sylvester et al., 2009). CIN decreased only average protozoal area, indicating CIN may inhibit cells in a different manner than MON. MON appeared to have a greater effect on decreasing motility, which may have inhibited cell function and viability. Previous research with *Paramecium* indicated that ionophores inhibited lysosome and digestive vacuole fusion as well as proteolysis, and it is likely that protozoa could be inhibited in the same manner (Fok, 1987). CIN decreased protozoal area, which may be due to an effect on the cell membrane. This observation is consistent with previous research by Gill and Holley (2004) in which cinnamaldehyde's inhibition of bacteria was associated with either disruption of membrane function or glucose utilization. The treatment interaction, in which CIN decreased MON's inhibition on average speed, was contrary to the original hypothesis of this study and may be related to how the inhibitors contacted the protozoa. This interaction was analogous to the interaction found by Ye (2013), in which MON and CIN did not have an additive inhibitory effect for decreasing protozoal counts or decreasing methane production.

CIN decreasing cell size and having an effect on the cell membrane may have decreased the ability of MON to contact the cell and cause its own inhibition on cellular components.

The ImageJ analysis with a lack of significant differences at 6 h post-feeding was attributed to protozoal viability by this time point. In future studies, it may be beneficial to shorten the time points of video analysis and data collection to 0, 2, and 4 h post-feeding in order to obtain more results from viable protozoa. Data obtained with a 2-h and 4-h time point may contribute further insight into how inhibitors affect motility.

Compared with more subjective protozoal motility methods, the ImageJ method will improve efficiency, accuracy, and/or precision for future studies as was originally hypothesized. This study found that defining parameters for detection prior to processing each video was the most effective way of decreasing error using ImageJ. Further exploration in the use of ImageJ for protozoal motility analysis should focus on this pre-processing portion of detection rather than on deleting tracks once data are generated. Eliminating tracks after processing did not appear to be as simple or as effective. This post-processing method was less effective because tracks often jumped from a cell to a feed particle or from cell to cell. Each track's individual data cannot be altered post-processing to correct for this. The most effective way of improving accuracy within this program will be to decrease the amount of times a track jumps from one object to another and this will have to be done prior to processing the videos and generating data. In all, ImageJ is useful due to the large amount and different types of data that it is able to generate and also its ability to greatly decrease the amount of subjectivity involved with previous methods of motility analysis.

Despite the benefits of ImageJ, subjective scoring on a 0-5 scale may remain useful for some studies. The subjective scoring took approximately 1/6 the amount of time to process videos, and may be most useful when general motility data are needed for future projects.

CONCLUSION

MON was found to be the strongest and most consistent protozoal motility inhibitor. The main effect of MON decreased protozoal distance traveled and average speed. CIN's main effect was on average protozoal area and was likely due to an impact on the cell membrane. ImageJ analysis was found to be useful for its large amount of data output as well as its ability to decrease subjectivity in motility analysis. Further work on pre-processing methods to improve cell detection would be beneficial to continued improvement of this program's accuracy for future studies. Due to the length of video processing time required with ImageJ, subjective motility analysis may remain useful for gathering general motility data.

REFERENCES

Benchaar, C., & Greathead, H. (2011). Essential oils and opportunities to mitigate enteric methane emissions from ruminants. Anim. Feed Sci. Technol., 166, 338-355.

Fok, A. K., Ueno, M.S. (1987). Ionophores and weak bases inhibit phagolysosomal proteolysis in Paramecium. Eur J Cell Biol. 45(1):145-150.

Gill, A. O. & Holley, R. A. (2004). Mechanisms of Bactericidal Action of Cinnamaldehyde against Listeria monocytogenes and of Eugenol against L. monocytogenes and Lactobacillus sakei. Appl. Environ. Microbiol. 70(10): 5750-5755.

Hook, S. E., Wright, A.-D. G., & McBride, B. W. (2010). Methanogens: Methane Producers of the Rumen and Mitigation Strategies. Archaea, 2010, 1-11.

Hristov, A. N., Oh, J., Firkins, J. L, Dijkstra, J., Kebreab, E., Waghorn, G., Makkar, H. P. S., Adesogan, A. T., Yang, W., Lee, C., Gerber, P. J., Henderson, B., & Tricarico, J. M. (2013). Mitigation of methane and nitrous oxide emissions from animal operations: I. A review of enteric methane mitigation options. J. Anim. Sci., 91, 5045-5069.

Knapp, J. R., Laur, G. L., Vadas, P. A., Weiss, W. P., & Tricarico, J. M. (2014). Invited review: Enteric methane in dairy cattle production: Quantifying the opportunities and impact of reducing emissions. J. Dairy Sci., 97(6), 3231-3261.

Morgavi, D. P., Martin, C., Jouany J.-P., & Ranilla, M. J. (2011). Rumen protozoa and methanogenesis: not a simple cause-effect relationship. British Journal of Nutrition, 2011, 1-10.

Morris, D. L. (2015). *Effects of Feeding Stearic, Linoleic or Lauric Acid on Rumen Protozoal Fatty Acid Levels, Proportion of Living Cells and Motility*. Unpublished manuscript, Department of Animal Sciences, The Ohio State University, Columbus, OH.

Newbold, C. J., de la Fuente, G., Belanche, A., Ramos-Morales, E., & McEwan, N. R. (2015). The Role of Ciliate Protozoa in the Rumen. Frontiers in Microbiology. 6:1313.

Nikon® United States. Nikon® D-500 Camera. 2010.

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Schindelin, J., Arganda-Carreras, I., & Frise, E. et al. (2012). Fiji: an open-source platform for biological-image analysis. Nature methods 9(7): 676-682.

Schneider, C. A., Rasband, W. S., & Eliceiri, K. W. (2012). NIH Image to ImageJ: 25 years of image analysis. Nature Methods 9: 671-675.

Sylvester, J. T., Karnati, S. K. R., Dehority, B. A., Morrison, M., Smith, G. L., St-Pierre, N. R., & Firkins, J. L. (2009). Rumen protozoa decrease generation time and adjust 18s ribosomal DNA copies to adapt to decreased transfer interval, starvation, and monensin. J. Dairy Sci 92:256-269.

Tetratracker Plugin for Windows7 (64bit). Modified wormtracker.

VirtualDub for Windows 7 (64bit). GNU General Public License (GPL).

Van Soest, P. J. (1994). Nutritional Ecology of the Ruminant. (2nd Ed.). Ithaca, NY: Cornell University Press.

Wagner, B. (2014). *Determining the effect of protozoal inhibitors on protozoal cell concentration, cellular protein, and cell volume to improve livestock feed efficiency.* Unpublished manuscript, Department of Animal Sciences, The Ohio State University, Columbus, OH.

Ye, D. (2013). *Examining the effects of adding fat, ionophores, essential oils, and Megasphaera elsdenii on ruminal fermentation with methods in vitro and in vivo* (Doctoral dissertation). Retrieved from ProQuest, ProQuest Dissertations and Theses. (UMI No. 1647743740).