Evaluation of Inhibitors of Lysozyme and Peptidases as New Approaches to Control Growth of Rumen Protozoa

Thesis

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By

Chongwu Yang, M. A.
Graduate Program in Animal Sciences

The Ohio State University

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Thesis Committee:
Zhongtang Yu, Advisor
Jeffrey Firkins
Alejandro Relling
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Abstract

Rumen ciliates are the only predators in the rumen. They engulf ruminal bacteria and other microbes and use the microbial protein as a source of nitrogen and other nutrients after digesting the ingested bacteria using digestive enzymes contained in lysosomes. Both genes and mRNAs coding for lysozyme and peptidases including serine peptidase, metallopeptidase, and cysteine peptidase were found in *Entodinium caudatum* (*E. caudatum*), which represents the one of most predominant ruminal ciliates (Williams and Coleman, 1992). Lysozyme is a small and stable enzyme that lyses bacterial cells by breaking bacterial cell walls. Enzymatically, lysozyme hydrolyzes the 1,4-beta linkages between N-acetylmuramic acid and N-acetyl D-glucosamine residues in peptidoglycan. Serine peptidase, metallopeptidase, and cysteine peptidase are proteases that cleave specific peptide bonds of proteins. By degrading microbial proteins in the rumen, rumen ciliates drive intra-ruminal nitrogen recycling, which decreases nitrogen utilization efficiency in ruminant animals. It was hypothesized that inhibition of lysozyme and peptidases could inhibit growth of rumen *E. caudatum*, and thereby potentially improve nitrogen utilization by ruminants.

Experiments were designed to determine the effects of different chemical inhibitors of lysozyme and peptidases using *E. caudatum* as a model protozoan species. In the first *in vitro* experiment (chapter 3), two inhibitors each of lysozyme, serine peptidase, metallopeptidase, and cysteine peptidase at varying concentrations were
tested for their inhibition to *E. caudatum in vitro*. The results of the first *in vitro* experiment (chapter 3) show that all the tested inhibitors including imidazole, phenylmethylsulfonyl fluoride (PMSF), Pefabloc®, phosphoramidon disodium salt, bestatin, and iodoacetamide reduced (*p* < 0.01) counts of *E. caudatum* at 24 and 48 h incubation *in vitro*. The increased doses of each inhibitor all caused linearly (*p* < 0.01), quadratic (*p* < 0.01) and cubic (*p* < 0.01) decrease of *E. caudatum* counts. Based on the results of the first *in vitro* experiment (chapter 3), imidazole at 100 mM, PMSF at 3 mM, and iodoacetamide at 0.5 mM were selected for further evaluation. These inhibitors were also evaluated *in vitro* using *E. caudatum* cultures both individually and in two and three-way incubations. We examined the effects of these inhibitors on *E. caudatum* abundance, culture pH, ammonia concentration, the concentrations of total volatile fatty acids (VFA) and profiles, dry matter digestibility (DMD), and neutral detergent fiber digestibility (NDFD). Effects on diversity and structure of bacteria and archaea were investigated using amplicon sequencing analysis of 16S rRNA gene. All the three inhibitors, both alone and in combination decreased *E. caudatum* counts (*p* < 0.01) and ammonia concentration (*p* < 0.01), and the two- and three-way combinations were more effectively than the inhibitors individually for lowering ammonia concentration (*p* < 0.05). No differences (*p* > 0.05) were observed for total VFA concentrations, DMD, and NDFD among control and the treatments. However, the inhibitors affected the relative abundances of some rumen microbiota at phylum and genus levels. These results indicate that the inhibitors can effectively inhibit *E. caudatum* without adversely affecting feed digestion or fermentation even though they changed the bacterial populations in the cultures.
Dedication

Dedicated to the students at The Ohio State University
Acknowledgments

I would like to thank my advisor Dr. Zhongtang Yu first. I am so grateful for his guidance and patience. Dr. Yu always encourages me to think critically and never blamed me even I made simple and stupid mistakes. He taught me to think more critically and creatively and do experiments more independently. With his encouragement and mentoring, I gradually become more confidence doing experiments and grow my knowledge base and skill sets.

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Vita

May 2011…………………………..No.1 high school, Taian, Shandong

June 2015…………………………..B.S., Biotechnique, Department of Life Science, Shandong Agricultural University

Aug 2015 to present………………..Graduate Research Associate, Department of Animal Sciences, The Ohio State University

Publications


Fields of Study

Major Field: Animal Sciences
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<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>NH3</td>
<td>Ammonia</td>
</tr>
<tr>
<td>BCVFA</td>
<td>Branched chain fatty acids</td>
</tr>
<tr>
<td>CP</td>
<td>Crude protein</td>
</tr>
<tr>
<td>DMD</td>
<td>Dry matter digestibility</td>
</tr>
<tr>
<td>DM</td>
<td>Dry matter</td>
</tr>
<tr>
<td>EMPS</td>
<td>Efficiency of microbial protein synthesis</td>
</tr>
<tr>
<td>FAB</td>
<td>Fluid-associated bacteria</td>
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<tr>
<td>GC</td>
<td>Gas Chromatography</td>
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<tr>
<td>MCP</td>
<td>Microbial crude protein</td>
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<tr>
<td>NDF</td>
<td>Neutral detergent fiber</td>
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<tr>
<td>NDFD</td>
<td>Neutral detergent fiber digestibility</td>
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<tr>
<td>NUE</td>
<td>Nitrogen utilization efficiency</td>
</tr>
<tr>
<td>N</td>
<td>Nitrogen</td>
</tr>
<tr>
<td>OTU</td>
<td>Operational Taxonomic Units</td>
</tr>
<tr>
<td>OM</td>
<td>Organic matter</td>
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</table>
Organic matter digestibility ................................................................. OMD

Particle-associated bacteria .............................................................. PAB

Phenylmethylsulphonyl fluoride ......................................................... PMSF

Phosphoramidon disodium salt ......................................................... PDS

Principal coordinates analysis......................................................... PCoA

Quantitative Insights Into Microbial Ecology .................................... Qiime

Rumen degradable protein ............................................................... RDP

Rumen undegradable protein ........................................................... RUP

Short-chain fatty acids .................................................................... SCFA

Volatile fatty acids .......................................................................... VFA
Chapter 1: Introduction

The rumen is the microbial habitat with abundant bacteria, archaea, viruses, fungi, and protozoa. Nutrient degradation including hydrolysis of lignocellulosic feeds and degradation of protein are accomplished by the collective degradative activities of rumen microorganisms (Santra and Karim, 2002). Cellulolytic bacteria, amylolytic bacteria, and fungi together determine the rate and extent of polysaccharide degradation, while rumen protozoa play a minor role in feed digestion (Similarities, 2014; Lee and Ha, 2000; Mendoza et al., 2014). This has been demonstrated in previous studies that compared feed digestibility in faunated (protozoa-carrying) and defaunated (protozoa-free) sheep. Firstly, rumen protozoa promote methane production due to their production of methanogenesis substrates and positive association with methanogens. Secondly, and more importantly, rumen protozoa prey on rumen bacteria and mediate wasteful intra-ruminal dietary nitrogen recycling (Jouany and Ushida, 1999). Therefore, rumen protozoa are believed to be primarily responsible for low nitrogen utilization efficiency (NUE) and excessive nitrogen excretion from ruminant animals. In addition, it is a long-standing interest to improve nitrogen utilization efficiency by inhibiting the growth of rumen protozoa, rather than by defaunation, using inhibitors.

Previous studies have evaluated various protozoal inhibitors, such as yucca saponin (Wang et al., 1999), tea saponin (Hu et al., 2005), and essential oils (Patra and Yu., 2012; Patra et al., 2015), for their efficacy to inhibit rumen protozoa. However, none of these are specific inhibitors, and often adverse effects on feed intake or
digestions are reported for these plant derivatives. There is a need to identify inhibitors that can specifically control rumen protozoa with little or no adverse effect on rumen function or ruminal microbiota. It was hypothesized that the unique digestive enzymes of rumen protozoa, such as lysosomal lysozyme and peptidases, could inhibit growth of *E. caudatum*. In this study, this hypothesis was tested by evaluating inhibitors of lysozyme and three families (serine peptidase, metallopeptidase, and cysteine peptidases) for their ability to inhibit *Entodinium caudatum*, one of the most predominant and bacterivorous protozoan species in the rumen. Promising inhibitors for each type of the enzymes were further investigated for their efficacy and their effects on feed digestion, fermentation, ammonia concentration, and relative abundance of rumen microbiota. The results were promising, and the inhibitors of those lysosomal enzymes may have potential to be used in control rumen protozoa to achieve improved nitrogen utilization efficiency.
Chapter 2: Review of Literature

Rumen ciliate protozoa

The quantification of protozoal biomass has been hampered by a suitable chemical marker (Firkins et al., 1998), however, the most predominant protozoa are ciliates at a density of approximately $10^4$ to $10^6$ cell counts per ml rumen fluid (Clarke, 1977). Ruminal ciliates are either oligotrichs or holotrichs, and their populations and proportions are primarily determined by the composition and the physical characteristics of diet as well as feeding frequency (Jouany, 1989). Holotrichs, with cilia covering their entire body, primarily utilize soluble carbohydrates including starch and sugar, whereas oligotrich ciliates with cilia found only in the mouth region, consume and ferment insoluble carbohydrates such cellulose and hemicellulose (Williams, 1986). Oligotrichs represent more than 95% of the total ciliate community, whereas holotrichs only account for less than 5% of the ciliate population in the rumen. However, due to their large cellular size, holotrichs can account for nearly 40% of the protozoal biomass (Williams, 1986). *E. caudatum* is one of the most predominant protozoan species in the rumen, and it shares similar morphological appearance and fermentative characteristics with other oligotrich ciliates on the rumen. *E. caudatum* population increased in ruminants when fed high amount of insoluble carbohydrates (Belecki et al., 2016).
In addition to utilizing sources of carbohydrates from diets to obtain energy, rumen protozoa engulf and digest bacteria by lytic enzymes enclosed in lysosomes for their core protein sources (Coleman and Laurie, 1974). A single protozoan cell in the rumen is able to take up $10^2$-$10^4$ bacteria per hour (Coleman, 1975). The rate of such engulfment can be affected by several factors including starvation, and the engulfment can be both selective and in-selective (Coleman, 1986). Coleman and Sandford (1979) found that one of the most predominant rumen protozoa, *E. caudatum*, ingests nearly all bacteria without any selection while other species like *Epidinium caudatum*, selects certain bacteria of rumen origin in *in vitro* experiments. And the amounts of protozoa consumed per protozoa is greater *in vitro* studies than in vivo studies. During engulfment, bacteria are engulfed and digested by protozoa rapidly, and the uptake of bacteria increased with the increasing initial concentration of bacteria in the culture (Coleman, 1964). Uptake of bacteria by protozoa decreases nitrogen utilization efficiency (NUE) and efficiency of microbial protein synthesis (EMPS) in the rumen. This has been demonstrated in several studies where defaunation, complete elimination of protozoa from the rumen, decrease ammonia concentration in the rumen (Koenig et al., 2000; Newbold et al., 2015). The importance of the rumen protozoa to the health of adult ruminants and growth of young ruminants has been debated since 1929 (Beck, 1929; Williams and Coleman, 1992). Some studies (Koenig et al., 2000) found that defaunation decreases ammonia concentration, indicating better dietary nitrogen utilization efficiency (NUE). However, despite reduced protozoa decrease intra-ruminal N recycle and elevated efficiency of microbial protein synthesis (EMPS) after feeding dietary fat (Firkins, 1996), benefits of defaunation are still remaining controversy. Defaunation of rumen decreases cellulose degradation rate, ammonia concentration, methane production, increase bacterial density but depress fiber
digestibility. However, studies of defaunation are mostly with nonlactating or low-producing animals (Hristov and Jouany, 2005). And suppression of rumen protozoa is a better way which increase efficiency of microbial protein synthesis (EMPS) without the negative impact of complete defaunation (Firkins et al., 2006). It was hypothesized that inhibiting rumen ciliates growth, rather than eliminating them, might be beneficial for nitrogen utilization efficiency (NUE) and avoiding decreasing fiber digestibility.

*Entodinium caudatum* is widely distributed in rumen fluids and its single cell was isolated from rumen as pure culture for *in vitro* experiment (Dehority, 2010). The optimum temperature for growth of *Entodinium caudatum* in the rumen is about 39°C (Williams and Coleman, 1992). Dehority (2005) found that *E. caudatum* is sensitive to rumen pH as other species of rumen ciliates. *Entodinium caudatum* would increase when pH rose from 5.5 to 6.0 and decrease as pH dropped to approximately 5.4 and eventually disappeared after exposed at pH 5.4 for an extended period of time (Dehority, 2005).

**The efficiency of nitrogen utilization**

The efficiency of nitrogen utilization (NUE) in the rumen is the key factor in ruminants in determining animal production and environmental impact (Calsamiglia et al., 2010) Ruminal ammonia concentration is a predicator of efficiency of dietary nitrogen conversion into microbial nitrogen (Firkins et al., 2007). Ruminants do not utilize dietary nitrogen as efficiently as non-ruminants, and more than half of dietary N is converted to ammonia, and large portion of ammonia (Calsamiglial et al., 2010). Ammonia is recycled back to the rumen from urea and utilized by rumen microorganisms. However, when ammonia is produced too rapidly in rumen which
exceeds the utilization by rumen microorganism, large portion of ammonia is excreted in urea and feces (Firkins, 2005; Ishler, 2004). When ammonia is released into the atmosphere, it reacts with other gases including sulfuric acid to form ammonium sulfate, which is harmful to the air quality (Firkins, 2005; Ishler, 2004). Under low dietary protein conditions, cattle are more likely to utilize nitrogen more efficiently especially for high production animals (Calsamiglia et al., 2010). However, the average NUE among ruminants is only around 25%, and low NUE not only increases production cost but also leads to environment pollution with excreted nireogen. Higher ammonia concentration in the rumen appears to be associated with lower NUE (Bach et al., 2005). Thus, ruminal ammonia concentration is one of the indicators for predicting NUE (Newbold et al., 2015).

There have been several comprehensive reviews (Bach et al., 2005; Calsamiglia et al., 2010; Jouany, 1996) on the relationship among rumen protozoa, bacteria, and NUE. This relationship was extended to defaunation and ammonia concentration in many studies and was corroborated in a recent meta-analysis (Newbold et al., 2015). Because rumen bacteria provide 50-80% of total absorbable protein for ruminants (Storm and Orskov, 1983), the bacterial population in the rumen is one of the most important factors affecting NUE. Rumen protozoa, including *E. caudatum*, reduce NUE by converting bacterial N to ammonia after engulfing rumen bacteria at a rate of 20-3000 bacteria per protozoan per hour (Coleman and Laurie, 1977; Bach et al., 2005; Virtanen, 1969). Another explanation of low NUE attributed to protozoa is that protozoa pass out of the rumen relatively slower than bacteria and therefore a relatively smaller proportion of protozoal protein can be utilized by ruminants (Williams and Coleman, 1992). The importance of rumen protozoa to ruminant health and the
development of ruminants has been debated since Becker (1929) first identified that ruminant animals could survive and live normally after defaunated. However, defaunation showed numerical positive influences on rumen function such as higher bacterial concentration, lower methane emission, increased microbial N flow, higher efficiency of microbial protein synthesis (EMPS), although several adverse effects were noted after defaunation such as lower organic matter digestibility (OMD) and neutral detergent fiber digestibility (NDFD).

Efficiency of microbial protein synthesis

Achieving efficient animal production and better animal performance requires attention to optimize the efficiency of microbial protein synthesis (EMPS) and NUE (Isher, 2004). Synthesis of microbial protein, expressed as grams of microbial N per unit of rumen available energy, in the rumen is the result of rumen microorganism metabolism. When protein requirement exceeds synthesis of protein caused by low EMPS especially for lactating cows, production and protein content of milk and meat can be reduced (Koenig et al., 2000; Reynolds and Kristensen, 2008).

The efficiency of microbial protein synthesis has been discussed in relation to the NUE. Bach et al. (2005) concluded that NUE in the rumen peaked around 69% when EMPS is 29 g of bacterial N/kg of fermented organic matter implying that high NUE can be achieved by optimizing EMPS. Rumen protozoa prefer engulfing bacteria and utilize the microbial protein as the primary protein source over using amino acids or protein directly from feeds (Bach et al., 2005; Sakanari et al., 1989). The role of microbial protein synthesis and degradation played by protozoa in the rumen remain unclear. The flow of protozoa from rumen to duodenum is low because of the
sequestration of protozoa on feed particles and the rumen epithelium (Hook et al., 2017). Sok et al. (2017) found that approximately 16.5% of microbial crude protein (MCP) arising from protozoal protozoa reach to duodenum, whereas 33% of MCP is from fluid-associated bacteria (FAB) and 50% particle-associated bacteria (PAB) by using rigorous screening and quantification approaches. Rumen Protozoa are not essential to the animal survival, defaunation (removal of protozoa) has been used to optimize rumen function and animal performance in research (Becker, 1929; Williams and Coleman, 1992). A meta-analysis done by Newbold et al. (2015) summarized and evaluated previous studies. The value of EMPS from different studies in defaunated animals differed; however, low ammonia concentration and high microbial protein flow are the most consistent of the effects after removing protozoa (Demeyerc and Van Nevel, 1979). The increase in overall EMPS observed in defaunated animals highlights the fact that elimination of rumen protozoa has positive effects on rumen function, animal performance as well as milk and meat production (Newbold et al., 2015). However, previous studies of defaunation are primarily for low-producing cows and non-lactating cows, and the contribution of protozoal protein to microbial protein under different dietary conditions for high-producing cows is not known (Firkins et al., 2007).

**Digestive enzymes**

Although ciliates possess the ability to utilize dietary carbohydrates, it is well established that ciliates engulf bacteria and use the microbial protein as their major protein sources (Coleman and Laurie, 1974). Digestive enzymes, such as lysozyme and proteases, are found in protozoa (McKerrow, 1993). Rumen protozoa, especially oligotrichs, are very bacterivorous and can engulf large numbers of rumen bacteria,
exerting a significant effect on NUE and EMPS. Two types of enzymes, lysozyme and peptidase produced by protozoa, are contained in lysosomes. Lysosomes are organelles in the cytosol of rumen protozoa containing digestive enzymes, including lysozymes, peptidases, glycoside hydrolases, lipase, etc. The mechanism or process of engulfment of bacteria by rumen protozoa is not well understood, but it is thought to be similar as that of environmental ciliates. After the first step of adherence of microbe to protozoa, the cell membrane of the protozoa at the oral area is invaginated, and a phagosome is successfully formed. Upon fusion with a lysosome, a phagolysosome forms (Williams and Coleman, 1992). Bacteria in the phagolysosome are thought to be lysed by the digestive enzymes, including lysozyme and peptidase and lipase. Previous studies showed that hydrolytic enzymes, including lysozymes and several types of peptidases derived from lysosome, are released from protozoa in the suspension of *in vitro* incubation, and some bacterial debris could be seen after adding protozoa, suggesting that protozoa produce lysozymes and peptidase that can digest bacteria after engulfment (Muller, 1972; Coleman and Laurie, 1974; Ling, 1990). Direct evidence of lysozyme, peptidases, glycoside hydrolases, and lipase was obtained from a recent genomic and transcriptomic studies (unpublished data of our lab).

Regarding the effects of lysozyme effects on bacterial digestion, some old literature reported degradation of bacterial cell wall by hydrolyzing the beta-1, 4-linkage between N-acetylmuramic acid and N-acetyl-D-glucosamine residues of peptidoglycan, the major compound of bacterial cell wall (Ghysen and Strominger, 1966). Coleman and Laurie (1974) isolated lysozyme released from three *Epidinium* species from the rumen. Bukharin and Nemtseva (2000) determined that some bacterial strains were resistant to lysozymic activity and survived from protozoa *in vitro*. 
Proteases are another type of digestive enzymes produced by rumen protozoa. They can be classified into a number of types including serine peptidase, metallopeptidase, and cysteine peptidase (Coleman and Laurie, 1974; Muller, 1972). Brock et al. (1982) confirmed the presence of serine peptidase, metallopeptidase, and cysteine protease by adding their inhibitors to protozoal cultural supernatant in-vitro. Serine peptidase in the rumen is found ubiquitous in protozoa after engulfing bacteria. Serine peptidase hydrolyzed the peptide bonds of protein or peptides containing special serine residues (Hedstom, 2002). The mechanism of serine protease involves catalyzing the hydrolysis of ester or amide (Wong and Whitesides, 1994). Serine protease genes are highly expressed in protozoa and have been successfully isolated from rumen protozoa in previous studies (Sakanari et al., 1989). Metallopeptidases were isolated from protozoa and act as important enzymes (Rawling and Barret, 1995). Metallopeptidases probably aid digestion of bacteria by affecting the cell surface and extracellular matrix (Baker, 2002). Metal ions are needed to bind the substrate to the active site of metallopeptidase and catalyze hydrolysis of side chains of peptides. Cysteine protease plays a vital role in the digestion of rumen protozoa by catalyzing a nucleophilic cysteine thiol in catalytic triad formed by the cysteine and histidine residues of protein (Grzonka et al., 2001). Cysteine protease has been described as the major protease in rumen protozoa that contributes to more than half of endogenous proteolytic activity (Barret et al., 1977). Cysteine peptidases have been successfully isolated from bacteria, plants as well as rumen protozoa (Otto and Schirmeister, 1997). Activities of protease produced by protozoa are affected by pH and temperature in a similar fashion as other enzymes. Despite the impact on protozoal digestion, the roles of protease in rumen protozoa survival remain unclear.
Inhibitors of rumen protozoa

In any event, although defaunation appeared to show benefits in rumen functions and animal performance, it is noteworthy to observe that previous studies showed adverse effects, including decreased OM digestibility. Besides the adverse effects of defaunation, many chemicals added to eliminate ciliates are toxic or harmful to ruminants. Therefore, identifying inhibitors to control ruminal protozoa, instead of eliminating them, may minimize the adverse effects mentioned above of defaunation.

Natural and synthesized inhibitors have been tested both in vitro and in vivo to determine their effects on rumen protozoal growth (Firkins et al., 2005). However, these protozoa inhibitors, such as yucca saponin (Wang et al., 1999), tea tannin (Hu et al., 2005) and essential oils (Patra and Yu, 2012; Patra and Yu, 2015), also show adverse effects on rumen functions, decrease feed intake, and broadly impact ruminal bacteria. Considering the important role played by digestive enzymes produced by protozoa, specific inhibitors of lysozymes and peptidases may be explored to control rumen protozoa. Lysozyme inhibitors, imidazole and indole-3 propionic, bind to the center of the hydrophobic core of lysozymes, changing the conformation of the active site (Swan, 1972). Imidazole and its derivatives form the charge-transfer type between them and the indole moiety of the tryptophan residues present in the active sites of enzymes (Shinitzky et al., 1966). Imidazole and its derivatives are effective inhibitors, and previous studies have shown that imidazole and its derivative imidazole, inhibit the lysis of Micrococcus Lysodeikticus by lysozyme at a concentration of 0.01-0.4 M (Shinitzky et al., 1966). Two serine esterases, phenylmethylsulphonyl fluoride (PMSF) and Pefabloc®, with a potential of selective sulfonylation of serine peptidase, were first
found by Gold and Fahreny (1963). Aside from their role in inhibiting chymotrypsin-like serine protease, it is well known that PMSF and Pefabloc® inhibit serine protease purified from rumen ciliates (Brock et al., 1982). Phosphoramidon sodium salt (PDS) and bestatin, as cell membrane surface metallopeptidase inhibitors, can effectively inhibit thermolysin and zinc metallopeptidase (Baker, 2002; Kitagishi and Hiromi, 1984). However, the inhibitory effect of PDS and bestatin on metallopeptidase purified from rumen ciliates has not yet been confirmed. Because cysteine peptidase is responsible for 60 to 70% of endogenous proteolytic activity in rumen ciliates, inhibition of cysteine peptidase can achieve effective inhibition of ruminal protozoa. Because iodoacetamide can successfully and quickly alkylate cysteine residues to block cysteine peptidase, iodoacetamide has been used for cysteine residue protector and as a reagent of cysteine modification since the early 1930’s (Chalker et al., 2009).

Previous studies have tested the effects of indo-3 propionic, imidazole, PMSF, Pefabloc®, except PDS and bestatin, in enzymes purified from rumen ciliates. However, no previous studies have ever tested their effects on ciliates and bacteria growth. I hypothesized that inhibition of these digestive enzymes will effectively inhibit cell counts of ruminal E. caudatum. This hypothesis was tested in this thesis research using E. caudatum as the model rumen protozoa in vitro. The effects of the inhibitors on feed digestion, ammonia concentration, pH, VFA concentration, and bacterial and archaeal communities were also examined.
Chapter 3: *In vitro* Experiments to Test the Effects of Inhibitors of Lysozyme and Peptidases on *E. caudatum* Growth

Abstract

Nitrogen utilization inefficiency not only increases feed costs and decreases animal production, but also causes air and soil pollution by increasing ammonia output from excreted urine and manure. Rumen microbes possess the ability to utilize dietary nitrogen to synthesize microbial proteins, which contribute up to more than half of the total absorbable proteins of ruminants. Rumen ciliates, with their abilities to engulf and digest bacteria, were assumed to be critical for decreasing nitrogen utilization efficiency (NUE). I hypothesized that rumen protozoa could be inhibited by inhibitors of lysozyme and peptidases and decrease ammonia genesis. The first objective of the *in vitro* experiment was to identify promising inhibitors of lysozyme, cysteine peptidase, serine peptidase, and metallopeptidase, each at three concentrations, can effectively inhibit the growth of *Entodinium caudatum*. One or two specific inhibitors each enzyme type was tested at one selected concentrations. The experiment was *in vitro* using a single-species of the protozoal culture of *E. caudatum* in SP medium. In order to exclude the influences of solvents of a few inhibitors, same volumes of solvents were added to each treatment. All cultures were incubated at 39°C, and the protozoa abundance was enumerated by microscopic counting at 24 and 48 h post incubation. All six inhibitors (imidazole,
phenylmethylsulphonyl fluoride, Pefabloc®, bestatin, iodoacetamide, phosphoramidon disodium salt) decreased ($p < 0.01$) protozoa abundance at very low concentration. Some of these inhibitors may be further evaluated for their efficacy and effect on rumen fermentation and other microbes.

**Introduction**

Dietary protein is the most expensive feed ingredient of food animals, including both ruminant and non-ruminant animals. Besides affecting the profitability of animal producers, nitrogen utilization efficiency (NUE) also determines the amount nitrogen discharged into the environment. Low NUE both increases livestock production cost but also cause pollution of the environment (Reynolds and Kristensen, 2008). However, NUE in ruminants is much lower and variable than in non-ruminants because of the microbial metabolism in the rumen (Kohn et al., 2005; Calsamiglia et al., 2010). Up to 60% of the ingested dietary protein is metabolized by rumen microbiota and become microbial protein. Microbial protein is the major direct nitrogen source, contributing to 60-70% of total degradable protein that ruminants need. Rumen ciliates play an important role in low NUE because they engulf various ruminal bacteria and digest their cellular protein into oligo peptides and free amino acids, both of which can be rapidly fermented by some ruminal bacteria (Bach et al., 2005; Virtanen, 1969), especially hypo-ammonia producing bacteria (HAB), into ammonia and short-chain fatty acids (SCFA) (Koenig et al., 2000). Although protozoa make up nearly half proportion of the rumen microbial biomass low producing ruminants, the flow of rumen protozoa from reticulo-rumen to duodenum is quite low (Harrison et al., Leng et al., 1980; Hook et al., 2017). Therefore, rumen protozoa are the primary cause of wasteful
intra-ruminal nitrogen recycling. Although there are exceptions, defaunation decreases rumen ammonia concentration and increased NUE (Newbold et al., 2015). Although lower ammonia concentration has been confirmed in defaunated sheep (Newbold et al., 2015), adverse effects including decreased NDF digestibility and duodenal fatty acids flow have also been observed. Besides, chemicals used for defaunation are toxic. Therefore, controlling protozoal growth instead of killing them totally may minimize the adverse effects caused by defaunation.

Numerous previous studies have evaluated different approaches in controlling the populations of ruminal protozoa, including plant secondary metabolites and lipids, none of them could specifically and consistently control the population of rumen ciliates (Hu et al., 2005; Patra et al., 2012; Patra and Yu, 2012; Patra and Yu, 2015; Wang et al., 1999). Because lysozymes and peptidases are necessary to digest engulfed bacteria, both Gram-positive and Gram-negative bacteria, inhibition of these two types of enzymes can specifically inhibit protozoal growth. In this in vitro experiment, six inhibitors for lysozyme, cysteine peptidase, serine peptidase, and metallopeptidase were tested using E. caudatum in vitro. I hypothesized that E. caudatum growth could be inhibited by the inhibitors. The objective of the experiment was to determine the effect of lysozyme and peptidases, each at 3 doses, on inhibiting growth of E. caudatum.

**Materials and Methods**

**Inhibitors selection**

A literature search was conducted with the key words “lysozyme” and “inhibitors” and “protozoa” and “peptidase” or “protease” and “inhibitors” and
“protozoa” using PubMed and found no published studies. Then literatures were searched again by not including the keyword “protozoa” and a few inhibitors were found that have been used in enzymological studies. After evaluating their inhibitory efficacy in the literature and commercial availability, the following six different inhibitors were selected for primary screening: imidazole (a lysozyme inhibitor), phenylmethylsulphonyl fluoride (a serine peptidase inhibitor), Pefabloc® (a serine peptidase inhibitor), phosphoramidon disodium salt (a metallopeptidase inhibitor), bestatin (a metallopeptidase inhibitor), and iodoacetamide (a cysteine peptidase inhibitor). To identify the effects of different doses of promising inhibitors, each of the inhibitors was tested at low, medium and high doses with 3 replicates per dose: imidazole at 13.5, 27, 54 mg/ml; phenylmethylsulphonyl fluoride at 0.2, 0.4, and 0.8 mg/ml; Pefabloc® at 0.5, 1, and 2 mg/ml; phosphoramidon disodium salt at 0.006, 0.03, and 0.12 μg/ml; bestatin at 0.01, 0.05, and 0.1 mg/ml; iodoacetamide at 0.018, 0.09, and 0.45 μg/ml. These concentrations were selected based on the effective concentrations in enzymological reactions, with the low and high concentration being below and above the enzymatically effective concentrations, respectively. Each concentration was tested in vitro in three replicates. A control containing no inhibitor was also included in parallel in three replicates. A separate stock solution was prepared for each inhibitor. Phenylmethylsulphonyl fluoride is not water soluble, so it was dissolved in absolute ethanol. To rule out the solvent effect, the same volume of the ethanol was added to the control.

*SP medium*
The SP media (Dehority, 1993) was prepared and used to test the growth of *Entodinium caudatum* prior to the experiment. The composition of the SP medium (g/l) was as follows: mineral mix SP-1 (20 g/l K$_2$HPO$_4$), mineral mix SP-2 (16 g/l KHPO$_4$, 4 g/l NaCl, 0.212 g/l CaCl$_2$·H$_2$O, 0.154 g/l MgSO$_4$), clarified rumen fluid, 6% (w/v) NaHCO$_3$, 3% (w/v) cysteine·HCl, 0.1% resazurin and dH$_2$O. SP medium was autoclaved at 121°C for 20 min and let cool down to room temperature after preparation. SP media was kept for overnight at 39°C to check for contamination and oxidation.

**Preparation of Entodinium Caudatum culture**

The rumen ciliate *E. caudatum* culture, containing no other rumen protozoa, was activated from frozen stock by incubation in 39°C with SP media. The *E. caudatum* was cultured in glass bottles under CO$_2$ atmosphere, with each tube sealed with a rubber stopper. The cultures of *E. caudatum* were fed 0.12 ml feed suspension per 10 ml culture daily. The feed contained (g/l): 0.3 g ground wheat, 0.1 g ground grass hay, and 0.1 g ground alfalfa. The culture volume was enlarged by adding an equal volume of fresh SP media every 3 to 4 days of incubation at 39°C. Counts of *Entodinium caudatum* were checked before each dilution.

**Evaluation of the inhibitors in vitro**

The *E. caudatum* culture prepared above was inoculated into SP medium containing individual inhibitor at a preselected concentration in autoclaved 16×150 mm culture tubes. Each tube contained 2.4 ml SP media and 0.6 ml *E. caudatum* culture. The headspace of the tubes was O$_2$-free. The tubes were incubated at 39°C for 24 and
48 h and were fed 0.12 ml protozoal feed daily. One subsample (0.3 ml) was collected from each tube at 24 and 48 h of incubation, fixed with a 1 ml formalin fixative (50% formalin and glycerol) containing 10 μl brilliant green (catalog B67456), and save for microscopic counting. The growth of *E. caudatum* was estimated by microscopic counting at 100x magnification (Dehority, 1993) using a counting chamber (Hausser Scientific, catalog #3800).

**Statistical analysis**

Data were analyzed using the MIXED model procedure of SAS (SAS Institute Inc., Cary, NC v9.4): $Y_{ijk} = \mu + D_i + H_j + DH_{ij} + e_{ijk}$, continuous variable, $\mu$ is the overall *E. caudatum* population mean, $D_i$ is the fixed effects of the $i$th inhibitors dose ($i = 1, 2, 3, 4$), $H_j$ is the fixed effect of $j$th hour ($j = 1, 2$), and $e_{ijk}$ is the residual error. $DH_{ij}$ is the fixed effect of the interaction of $i$th inhibitor doses and $j$th hour. Orthogonal polynomial contrasts were used to analyze the linear, quadratic, and cubic effects of the increasing doses of each inhibitor on *E. caudatum* counts.

**Results**

The increased doses of each inhibitor all caused linear ($p < 0.01$), quadratic ($p < 0.01$) and cubic ($p < 0.01$) decrease of *E. caudatum* counts (Table 3.1). There was no significant interaction for inhibitor doses of imidazole ($p = 0.33$), PMSF ($p = 0.82$), Pefabloc® ($p = 0.06$), bestatin ($p = 0.80$) by time except phosphoramidon disodium salt.

Compared to the control without inhibitors, the highest doses (i.e., 54.46 mg/ml imidazole, 0.80 mg/ml phenylmethylsulphonyl fluoride, 0.45 mg/ml iodoacetamide, 2.0
mg/ml Pefabloc®, 0.10 mg/ml bestatin, and 0.12 mg/ml phosphoramidon disodium salt) decreased in *E.* cell counts by 96.9%, 95.9%, 94.9%, 95.9%, 75.4%, and 80.6%, respectively at 24 h and by 97.9%, 96.8%, 94.7%, 96.8%, 82.1%, and 82.1% respectively, at 48 h (calculated in Table 3.1). Thus, imidazole, phenylmethylsulphonyl fluoride, Pefabloc®, and iodoacetamide could inhibit *E. caudatum* more effectively and be used as specific inhibitors.

**Discussion**

All the digestive enzymes of rumen ciliates are contained inside of lysosomes to avoid self-destruction of cellular components (Khanna and Yadav, 2004). Lysozyme degrades the peptidoglycan of the cell wall of bacteria, while serine peptidase, metallopeptidase, and cysteine are the major enzymes that hydrolyze proteins (McKerrow, 1993; Muller 1972; Coleman and Laurie 1974; Ling, 1990). As hypothesized, inhibition of the lysozyme, peptidases, and peptidases (cysteine, serine, or metallo types) all substantially (by greater than 80% and up to 97%) inhibited the growth of *E. caudatum*. These results indicate that *E. caudatum* depends on these digestive enzymes for the acquisition of essential nutrients and that engulfed ruminal bacteria and other microbes are the sources of these essential nutrients.

The tested inhibitors inhibited *E. caudatum* cell counts to different degrees. The lysozyme inhibitor, imidazole, inhibited *E. caudatum* the most, followed by the inhibitors of cysteine peptidase (iodoacetamide) and serine peptidase (Pefbloc® and phenylmethylsulphonyl fluoride). The lysozyme inhibitor had probably prevented *E. caudatum* from lysing the engulfed bacteria and thus accessing the cellular proteins, whereas inhibitors of cysteine peptidase and serine peptidase probably did not prevent
bacterial lysis but inhibited hydrolysis of the bacterial proteins. Phosphoramidon disodium salt and bestatin, both of which are metalloprotease inhibitors, were less effective than the other inhibitors. Metallopeptidase is mainly responsible for digesting the proteins in cell surface (Baker, 2002), and the results might suggest that metallopeptidases are less important in digesting engulfed bacteria compared with lysozyme, serine peptidase, and cysteine peptidase.

Different inhibitors can potentially affect other aspects of the *in vitro* fermentation and microbes. In the first *in vitro* experiment, the goal was to screen available inhibitors for further evaluation and thus no other analysis was performed. By comparison of inhibitory effects and cost, imidazole, PMSF, and iodoacetamide were selected for further evaluation in Chapter 4.
<table>
<thead>
<tr>
<th>Treatment</th>
<th>Concentration</th>
<th>SEM</th>
<th>P value</th>
<th>Linear</th>
<th>Quadratic</th>
<th>Cubic</th>
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<td>Imi, mg/ml</td>
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<td>3493</td>
<td>2038</td>
<td>873</td>
<td>118</td>
<td>&lt; 0.01</td>
</tr>
<tr>
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<td>27656</td>
<td>2038</td>
<td>1164</td>
<td>582</td>
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<td>2620</td>
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<td>445</td>
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<tr>
<td>48h</td>
<td>27656</td>
<td>5822</td>
<td>2038</td>
<td>873</td>
<td>145</td>
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</tr>
<tr>
<td>pefabloc®, mg/ml</td>
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<tr>
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<td>1747</td>
<td>1164</td>
<td>394</td>
<td>&lt; 0.01</td>
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<tr>
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<td>1456</td>
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<td>873</td>
<td>168</td>
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<tr>
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<td>0.1</td>
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<td>10771</td>
<td>6987</td>
<td>708</td>
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</tr>
<tr>
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<td>9174</td>
<td>4949</td>
<td>539</td>
<td>&lt; 0.01</td>
</tr>
<tr>
<td>Iodo, mg/ml</td>
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<td>0.09</td>
<td>0.45</td>
<td></td>
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<td>5822</td>
<td>2620</td>
<td>1456</td>
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<td>PDS, mg/ml</td>
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<tr>
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<td>291</td>
<td>&lt; 0.01</td>
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</table>

Table 3.1 E. caudatum counts in the presence of imidazole, PMSF, Pefabloc®, bestatin, iodoacetamide and PDS at 24h and 48h. (Continued)
Table 3.1: Continued

Data were analyzed using dose levels of 0, 13.5, 27, and 54 mg/ml for imidazole; 0, 0.2, 0.4 and 0.8 mg/ml for PMSF; 0, 0.5, 1, and 2 mg/ml for Pefabloc®; 0, 0.01, 0.05, and 0.1 mg/ml for bestatin; 0, 0.018, 0.09, and 0.45 mg/ml for iodoacetamide; 0, 0.006, 0.03 and 0.12 mg/ml for phosphoramidon disodium salt.

1 imidazole dose x time interaction ($p = 0.33$); PMSF dose x time interaction ($p = 0.82$); Pefabloc dose x time interaction ($p = 0.06$); bestatin dose x time interaction ($p = 0.80$); iodo dose x time interaction was ($p = 0.05$); PDS dose x time interaction ($p < 0.01$).

2 Imi, imidazole; PMSF, phenylmethylsulphonyl fluoride; pef, Pefabloc®; bet, bestatin; iodo, iodoacetamide; PDS, phosphoramidon disodium salt.

3 SEM, the standard error of the mean
Chapter 4: Effects of Selected Inhibitors of Lysozyme and Peptidases on *Entodinium caudatum*, Fermentation and Prokaryotic Community *in vitro*

Abstract

Bacteria, protozoa, and fungi cohabitate the rumen and play important roles in affecting NUE. *Entodinium caudatum*, by engulfing and digesting bacteria, can affect ammonia concentration, VFA concentration and composition, DM and NDF digestibility and pH. The experiment (chapter 3) helped identify three inhibitors of (imidazole, phenylmethylsulphonyl fluoride, and iodoacetamide) that can dramatically inhibit *E. caudatum* *in vitro*. The objective of this study was to further evaluate the efficiency of the three selected inhibitors, both individually and in two- and three-way combinations, for their ability to inhibit growth of *E. caudatum* and effects on feed digestibility, VFA concentration, ammonia concentration, and pH *in vitro* experiment. The effects of the inhibitors on the microbiota in the *E. caudatum* cultures were also examined by amplicon sequencing of 16S rRNA genes. Imidazole (at 100 mM), phenylmethylsulphonyl fluoride (at 3 mM), and iodoacetamide (at 0.5 mM) all substantially inhibited *E. caudatum* at 24, 48, and 72h. The two- and three-combinations of these inhibitors tended to inhibit *E. caudatum* to a greater extent than the inhibitors individually. The inhibitors had little effect on culture pH. Ammonia concentrations were dramatically decreased (*p < 0.01*) by all the inhibitors and their
combinations. Total VFA concentrations and molar proportion of individual VFA remained not affected except isovalerate \((p = 0.01)\). The inhibitors did not decrease DM digestibility or NDF digestibility. However, the inhibitors affected the microbiota at phylum and genus ranks, with the relative abundance of \textit{Bacteroidetes} being decreased while that of \textit{Firmicutes} increased. The above effects were also noted for the genera \textit{Prevotella} and \textit{Streptococcus}. \textit{Proteobacteia} was increased by imidazole and its combination with phenylmethylsulphonyl fluoride. The inhibitors decreased or tended to decrease the relative abundance of \textit{Ruminobacter}. These results indicate that the inhibitors can effectively inhibit \textit{E. caudatum} without adversely affecting feed degradation or fermentation even though they changed the bacterial populations in the cultures.

\textbf{Introduction}

Nitrogen sources from the feed of ruminants are primarily used for microbial growth and protein synthesis (Storm and Orskov, 1983). Microbial protein synthesized in the rumen contributes to 40-90\% of the total protein that dietary cows need (Leng and Nolan, 1984). Rumen ciliates, such as \textit{Entodinium caudatum}, engulf bacteria, digest them by digestive enzymes such as lysozymes and peptidase, utilize a portion of the digested/degraded bacterial protein to synthesize their own protein, but most of the digested bacterial protein is further degraded to ammonia and short-chain fatty acids (SCFA). Only a small portion of the resultant ammonia can be used in microbial protein synthesis, resulting in decreased nitrogen utilization efficiency (Bach et al., 2005). This is largely attributable to the wasteful intra-ruminal nitrogen cycling caused by rumen protozoa.
Numerous studies have evaluated different approaches to controlling the population of rumen protozoa. However, some natural inhibitors such as saponins and essential oil tend to inhibit the growth of protozoa, but few of these inhibitors at any of the tested doses affect concentrations of ammonia (Patra et al., 2012; Patra and Yu 2015), indicating that NUE may not be effectively improved. By searching for previous literatures, one of the most predominant rumen ciliate species, *Entodinium caudatum*, produces lysozymes and various peptidases including serine peptidases, metalloprotease, and cysteine at a high level (Morgravi et al., 1996; Swan, 1972; Sakanari et al., 1989; Rawlings and Barret, 1995). Because lysozyme is required for lysing bacteria engulfed by rumen protozoa, and peptidases are responsible for proteolysis, I hypothesized that inhibition of these two types of enzymes will provide a new approach to control rumen protozoa.

In first *in vitro* experiment, it was found that inhibitors of these digestive enzymes did inhibit *E. caudatum in vitro* substantially. The objective of this experiment was to evaluate if the inhibitors of each enzyme have interaction in inhibiting *E. caudatum*. It was hypothesized that the selected specific inhibitors (imidazole, PMSF, iodoacetamide), both alone and in two- and three-way combinations, can efficiently inhibit *E. caudatum*, decrease ammonia concentration without affecting volatile fatty acids (VFA) concentration, dry matter digestibility (DMD), Neutral detergent fiber digestibility (NDFD). I also hypothesized that inhibitors could also affect bacterial and archaeal populations.
Materials and Methods

Medium, protozoal culture, feed, and in vitro incubation

The medium (SP medium), protozoal culture (E. caudatum), protozoal feed, and in vitro incubation were essentially the same as described in Chapter 3, with the following exceptions: The in vitro culture volume was increased to 10 ml each, with 2 ml E. caudatum inoculum 7.755 ml of SP medium.

Treatments with inhibitors

A total 8 treatments, including one control that received no inhibitor, were used to systematically evaluate one inhibitor each for lysozyme (imidazole, at 100 mM), serine peptidase (phenylmethylsulphonyl fluoride (PMSF) at 3 mM), and cysteine peptidase (iodoacetamide at 0.5 mM), both individually and in two- and three-way combinations (Table 4.1). Each treatment had six replicates. All the treatment cultures were incubated for 72 h with daily feeding. At 0, 24, 48 and 72 h, 0.3 ml subsample each was collected to enumerate E. caudatum cell counts under a microscope. At 72 h, the end of incubation, pH was recorded.

Fermentation analysis

At the end of the 72h incubation, two milliliters of each culture were collected into a microtube and centrifuged at 16,000 x g at 4°C for 10 minutes to collect the supernatant for analysis of VFA using GC (gas chromatography) (Pantoja et al., 1994) and of ammonia using a colorimetric method (Chaney and Marbach, 1962), while the pellets were preserved at -80°C for DNA extraction. The supernatants for VFA analysis
were mixed with one volume of 33% metaphosphoric acid first, filtered, and stored in GC vials at 4°C. Supernatant for ammonia analysis was stored at -20°C. The remaining cultures (8 ml) were strained through 50 μm Ankom bags and dried at 55°C for two days to determine dry matter digestibility (DMD). After DMD analysis, the filter bags were processed to determine the NDF (Van Soest et al., 1991).

**DNA extraction and metagenomic analysis of microbiota**

Genomic DNA was extracted from pelletes by using the method RBB+C (Yu and Morrison, 2004). The DNA quality was checked by agarose gel and quantified by Quant-iT™ dsDNA Assay Kit (Thermo Fisher Scientific). The DNA was further diluted to 5 ng/ul and sent to the Molecular Cell Imaging Center at The Ohio State University (Wooster, OH) to undergo Illumina Miseq sequencing following the 2 × 300 paired-end protocol. The V4-V5 hypervariable region (primers set 515F and 806R) of the 16S rRNA gene was amplified.

The sequencing data were analyzed using Quantitative Insights Into Microbial Ecology (Qiime) (Caporaso et al., 2010) as described previously (Kigerl et al., 2016). Bases with a quality score lower than 25 were removed, then the paired reads were joined by the fastq-join algorithm (Erik and Aronesty, 2011). Clean sequences shorter than 252 bp were removed. Chimera sequences were identified using ChimeraSlayer (Haas et al., 2011) and filtered out. Species-equivalent operational taxonomic units (OTU) were clustered with Qiime using the pick-open-reference method against the Silva_119_release reference sequences at 97 % similarity using the uclust algorithm (Edgar, 2010). Minor OTUs, those each represented by less than 0.5% of total sequences, were filtered out (Bokulich et al., 2010). The alpha diversity measurements
(including Chao1 estimate, Simpson and Shannon indices) were calculated by Qiime. To compare the similarity between the microbial community between samples, the distance matrix between the samples was calculated by the weighted-Unifrac algorithm and PCoA plot was drawn based on the matrix thereby.

**Statistical Analysis**

The data on *E. caudatum* counts were analyzed using the Mixed model procedure of SAS (SAS Institute Inc., Cary, NC v9.4): $Y_{ijk} = \mu + T_i + H_j + T_iH_j + e_{ijk}$, $\mu$ is the overall *E. caudatum* population mean, $T_i$ is the fixed effect of the treatments ($j = 1, 2, 3, 4, 5, 6, 7, 8$), $H_j$ is the fixed effect of jth hour ($k = 1, 2, 3$), and $e_{ijk}$ is the residual error. $T_iH_j$ is the fixed effect of the interaction of the ith inhibitor type and the jth hour. Data were also analyzed among fermentative characteristics (pH, ammonia concentration, VFA concentration, DMD, and NDFD) and relative abundances of individual microbial taxa for the 72 hour time point using SAS 9.4. Data of the alpha diversity measurements (including Chao1 estimate, Simpson and Shannon indices) after calculating by Qiime were also analyzed using SAS 9.4. Significant differences of *E. caudatum* counts, fermentative characteristics, relative abundances of microbial taxa were analyzed using Duncan’s multiple range tests. Significance was declared at $p \leq 0.05$, and tendency at $0.05 < p < 0.10$. 
Results

_Inhibition of E. caudatum and effects on fermentation characteristics_

There was significant interaction for treatments ($p < 0.01$) by time. All the three inhibitors, both alone and in combination, decreased ($p < 0.01$) _E. caudatum_ counts after 24 h, 48 h and 72 h _in vitro_ incubation compared with those of control (Table 4.1). Although there was significant effect of inhibitors on inhibiting _E. caudatum_ growth ($p < 0.01$) except PMSF+Iodo treatment ($p = 0.17$) from 24 h to 48 h _in vitro_ fermentation, _E. caudatum_ seems to have no further decrease from 48 h to 72 h ($p > 0.10$). _E. caudatum_ counts with supplementation of individual inhibitor and the two-way combinations did not show differences ($p > 0.10$) at any of the three incubation time (24, 48, 72 h), while _E. caudatum_ counts were decreased significantly at 48 h compared with _E. caudatum_ counts at 24 h ($p < 0.05$). The three-way combinations also showed the greatest decrease in _E. caudatum_ counts numerically. The two combinations of PMSF with imidazole or iodoacetamide reduce _E. caudatum_ significantly ($p < 0.05$) compared with PMSF alone, indicating only PMSF show additive effects.

Compared with the control, imidazole and its two- and three-way combinations increased ($p < 0.01$) the culture pH at the end of the _in vitro_ incubation, but the increase was only less than 0.4 pH unit (Table 4.2). All the inhibitors, both individually and in combinations, decreased ($p < 0.01$) ammonia concentrations compared with the control (Table 4.2). Imidazole decreased NH$_3$-N the most, but PMSF the least decrease numerically. The three-way combination of inhibitor decreased ammonia concentration the greatest, by 86.1%. There were no inhibitory effects on DMD and NDFD ($p > 0.05$) between the control and all the treatments (Table 4.3). No difference in DMD and NDFD was seen either among the inhibitors, including their combinations.
All treatments with the inhibitors, both individually and in combinations, had similar concentrations of acetate \((p = 0.31)\), propionate \((p = 0.35)\), butyrate \((p = 0.46)\), valerate \((p = 0.18)\), and total volatile fatty acids \((p = 0.54)\) as the control (Table 4.3). There was only a trend of increased isovalerate concentration \((p = 0.09)\) and acetate to propionate ratio \((p = 0.07)\) in the treatments compared to with the control. However, PMSF, iodoacetamide, imi + PMSF, imi + iodo, and PMSF+iodo resulted in significant increase \((p = 0.01)\) in isobutyrate concentration.

**Effect on the Microbiota in the in vitro E. caudatum cultures**

The proportions of bacteria and archaea, as represented by % of sequence assigned to each domain, were affected by several of the inhibitors (Table 4.4). Overall, the proportion of total bacteria was significantly increased \((p < 0.01)\), whereas that of archaea was decreased \((p = 0.02)\) by all the inhibitors except imidazole and imidazole plus phenylmethysulfonyl fluoride, both of which tended to decrease archaeal proportion.

Principle coordinates analysis (PCoA) of the microbiota based on taxonomic assignments of the sequences showed that imidazole, iodoacetamide, phenylmethylsulphonyl fluoride, and the combination of all the three inhibitors resulted in clusters that were separated from that of the control along PC1, which explains greater than 64% of total variation (Figure 4.1). However, all the binary combinations of the three inhibitors did not result in clear separation. The treatments were not clearly separated along PC2 that explains 11.64% of the variation.
Fourteen phyla of bacterial were detected by the 16S rRNA gene sequences (Table 4.5). Only four phyla were each represented by greater than 1% of total sequences. All the inhibitors except PMSF significantly decreased ($p < 0.01$) the relative abundance of *Bacteroidetes* but increased ($p < 0.01$) that of *Firmicutes* (PMSF and its combination with imidazole tended to increase *Firmicutes*) (Figure 4.2). Imidazole, PMSF, and their combinations tended to increase ($p = 0.01$) the relative abundance of *Proteobacteria*, whereas the other treatments tended to decrease ($p = 0.01$) the relative abundance of this phylum. *Synergistetes*, the fourth largest phylum, was not affected by imidazole but was decreased ($p < 0.01$) by the other inhibitors and their combinations. The minor phyla were also affected ($p < 0.01$), but only to small extents. *Euryarchaeota* was the only archaeal phylum detected in the cultures, and its relative abundance was deceased ($p = 0.02$) significantly by PMSF, iodoacetamide, imidazole plus iodoacetamide, and PMSF plus iodoacetamide, and the combination of all the three inhibitors (Table 4.5).

In total 33 genera of bacteria and 2 genera of archaea were detected in the *E. caudatum* cultures. Only eight genera of bacteria were each represented by greater than 1% of the total sequences in at least one of the treatment (Table 4.6). In the control cultures, *Prevotella* was the most predominant genus, followed by *Ruminobacter*. All the inhibitors and combinations, except PMSF alone, decreased ($p < 0.01$) the relative abundance of *Prevotella*, but the opposite was true for the *Streptococcus* ($p < 0.01$). The relative abundance of *Ruminobacter* was decreased ($p = 0.04$) significantly (by imidazole plus iodoacetamide, PMSF plus iodoacetamide, and the three-way combination) or tended to be decreased (by the three inhibitors individually or by imidazole plus PMSF). Of other minor genera, most of the inhibitors and their
combinations changed their relative abundance \( (p < 0.01) \), which remained less than 1% of the total sequence.

**Discussion**

The purpose of this study was to evaluate inhibitors of enzymes that are needed by *E. caudatum* to lyse and digest the engulfed bacteria. We focused on their effect on feed substrate digestion, fermentation, and microbiota because they are all important to rumen functions.

*Effect of the inhibitors on feed substrate digestion and fermentation characteristics of the E. caudatum culture*

Utilization of nutrients is dependent on the collective activities of rumen bacteria, archaea, fungi, and protozoa. Defaunation improves rumen fermentation by increasing nitrogen and energy utilization efficiency, but it can cause some adverse effects such as low digestibility of organic matter (OM), milk lactose, and body weight gain (Koenig et al., 2000; Newbold et al., 2015). The inhibitory effects of imidazole, PMSF, and iodoacetamide and their combinations on *E. caudatum* were in agreement with many other inhibitors described previously such as tea saponin (Hu et al., 2005) and steroidal saponins from *Yucca schidifera* (Patra et al., 2012; Wang et al., 2000). However, compared to the control, all the tested inhibitors decreased *E. caudatum* to a much greater magnitude than the plant extracts, by 89.63% by imidazole (Imi, an inhibitor of lysozyme), 78.28% by phenylmethylsulphonyl fluoride (PMSF, an inhibitor of serine peptidase), 88.65% by iodoacetaminde (Iodo, an inhibitor of cysteine
peptidase), 89.63% by Imi+PMSF, 88.65% by Imi+Iodo, 86.89% by PMSF+Iodo, and 92.17% by Imi+PMSF+Iodo after 72 h incubation. These results showed that the selected inhibitors are very effective in inhibiting or controlling the growth of *E. caudatum*. Consistent with the results of the first *in vitro* experiment, the lysozyme inhibitor is the most inhibitory. No significant differences between inhibitors alone and combinations except PMSF suggest that any of the three types of enzymes is essential for *E. caudatum* to obtain the nutrients required for its survival and any of these enzymes can be a target to inhibit and control *E. caudatum*. These inhibitors may be able to inhibit the growth of other types of rumen protozoa, but further experiments are needed to test that possibility.

Among major rumen bacteria, Gram-positive bacteria such as *Ruminococcus*, *Butyrivibrio*, and *Pseudobutyrovibrio*, and Gram-negative bacteria including *Fibrobacter* and *Prevotella* are recognized as fibrolytic bacterial species, probably playing a major role in fiber degradation in the rumen (Koike and Kobayashi, 2009). Previous studies have also suggested that *Ruminococcus albus* and *R. flavefaciens* are particularly important cellulolytic bacteria (Pope, 2011; Mitsumori and Sun, 2008). In the present study, DMD and NDFD were not decreased (*p* > 0.05) by any of the inhibitors, either individually or in combination, indicating that the inhibition of the lysozyme and peptidases by the test inhibitors did not adversely inhibit fiber degradation. Among the rumen protozoa, *Epidinium*, *Polyplastron*, and *Eudiplodinium*, all of which belong to the family *Ophyroscoleidae*, are cellulolytic, while *Entodinium* is only hemicellulolytic (Takenaka et al., 2004). Compared to *Epidinium*, *Entodinium* is less capable of xylan degradation (Williams and Coleman, 1992). The lack of depression of DMD and NDFD when *E. caudatum* was inhibited suggests that the
contribution of *Entodinium* to fiber digestion is minimal or can be compensated by other microbes in the *in vitro* cultures. It should be noted that these inhibitors do not directly inhibit fiber degradation by rumen protozoa because they only inhibit bacterial cell lysis and protein hydrolysis. Given that defaunation can decrease fiber digestibility (Arakaki et al., 1995), inhibition of rumen protozoa by these inhibitors may alleviate that adverse effect on fiber digestion.

Rumen pH is a general indicator of feed quality and digestibility, saliva secretion, VFA concentration, and ammonia concentration. Normal ruminal pH varies between 5.5 to 7.3 and many rumen microbes may lose their vigor when pH is below 5.5 (Dehority, 2005). Ruminal pH is an important factor for sustaining a normal internal environment of the rumen, and low pH usually causes subacute ruminal acidosis (Ghorbani et al., 2000). Besides, catalytic activities of enzymes produced by rumen microorganisms decrease rapidly when pH values fall below 6.0 reducing ENU. The optimal ruminal pH for rumen *E. caudatum* growth is between 5.6 to 6.9, while the optimal ruminal pH for cellulolytic bacteria to grow is from 6.1 to 6.9. Therefore, we monitored the pH of the *in vitro* *E. caudatum* cultures to assess the effect of the inhibitors on fermentation.

The average pH of the *in vitro* *E. caudatum* cultures ranged from 6.68 to 7.06 in this study, which lies in the normal pH range for rumen microbiota. The values were relative higher (0.4 pH unit or less) compared with those reported in other *in vitro* studies (Patra et al., 2012; Patra and Yu, 2015; Patra and Yu, 2012). Because VFA concentration is the major factor determining the pH (Khafipour and Krause, 2009; Krause and Oetzel, 2015), the reason for the relatively high pH is that the *E. caudatum* cultures had lower VFA concentration (discussed below) than those reported in those
in vitro ruminal cultures. Among the treatments, imidazole and its two- and three-way combinations resulted in higher pH. The result could be attributed to imidazole, but the reason is not known.

Ammonia is an intermediate of ruminal nitrogen metabolism, and its concentration is determined by the rate of ammonia genesis (mainly from amino acid fermentation and urea hydrolysis) and rate of assimilation into microbial protein (Busquet, et al., 2005). Therefore, a high ammonia concentration indicates low EMPS and a low ammonia concentration accompanied with defaunation reflects improved ENU (Newbold, et al., 2015). Consistent with the results of decreased abundance of E. caudatum and defaunation studies (reviewed by Newbold et al., 2015), the ammonia concentrations were much lower in the E. caudatum treated with all the inhibitors, both individually and in combination. These results indicate that all the three inhibitors decreased deamination from amino acids. Theoretically, both decreased protein hydrolysis and subsequent amino acid fermentation can decrease ammonia concentration in vitro. Because the inhibitors used in the present study inhibit lysis of bacterial cells (the lysozyme inhibitor imidazole) or protein hydrolysis (the inhibitor of serine peptidase PMSF and the inhibitor of cysteine peptidase iodoacetamide), the observed low ammonia concentration in the E. caudatum cultures resulted from decreased protein hydrolysis. The results suggest that inhibition of the lysozyme and peptidases, at least cysteine peptidases and serine peptidases, can potentially improve ENU in ruminants.

The concentrations of total VFA and individual VFAs were lower in the E. caudatum cultures, irrespective the addition of the inhibitors, than those observed in in vitro rumen fermentation cultures (Patra et al., 2012; Patra and Yu, 2015; Patra and Yu,
2012). This might be explained by the low density of microbes of and the limited amount of feed added to the *E. caudatum* cultures. Except for the concentration of isobutyrate, a BCVFA, the inhibitors, individually or in combinations, did not affect the VFA concentrations. This is consistent with the lack of effect on the digestibility of DM and NDF. The acetate: propionate ratio was high in all the *E. caudatum* cultures, including the control culture. The acetate : propionate ratio was increased numerically by imidazole, PMSF, and their combination. The combination of imidazole and iodoacetamide also tended to increase the acetate : propionate ratio numerically. High acetate : propionate ratio is generally associated with high fiber diet. The feed fed to the *E. caudatum* cultures contained high fiber and low starch to slow down growth of the bacteria. The high acetate : propionate observed in all the *E. caudatum* cultures was probably attributed to the high fiber fed. The microbiota in the *E. caudatum* was probably very different from that in the rumen or in vitro ruminal cultures, and such microbiota difference could also be attributed to the high acetate : propionate ratio. However, most of the inhibitors and their combinations increased the concentration of isobutyric acid. Isobutyric acid can be produced in rumen by oxidative deamination and decarboxylation of valine (Daniel and Eldon, 1986). It remains to be determined if the increase of isobutyrate is derived from enhanced oxidative deamination and decarboxylation of valine. Valerate is produced mainly from fermentation of carbohydrates and from amino acids such as proline (Daniel and Eldon, 1986). The lack of changes of valerate suggests that supplementation of the inhibitors might not alter the metabolism of carbohydrate and proline that leads to valerate production. Previous study has found that supplementation of isoacids could increase milk production, microbial synthesis and enhance the growth of cellulolytic and hemicellulolytic microorganisms in the rumen (Daniel and Eldon, 1986). The increase of isobutyrate, as
an isoacid, might facilitate protein synthesis and fiber digestion. Further experiments are needed to analyze the effects of the tested inhibitors on microbial protein synthesis and fiber digestion.

**Effect of the inhibitors on the microbiota of the E. caudatum culture**

Rumen protozoa were thought to have association with and prey on select bacterial and archaeal populations based on results from studies using defaunated sheep (Mosoni et al., 2011; Morgavi et al., 2006; Xia et al., 2014). I hypothesized that the inhibitors could affect the bacterial and archaeal populations, directly or indirectly, of the *E. caudatum* cultures. Therefore, I analyzed the microbiota of the *E. caudatum* cultures. The inhibitors decreased the relative abundance of archaea, possibly due to decreased hydrogen production when *E. caudatum* was inhibited. Alternatively, the inhibitors could directly inhibit archaea, which can be tested using cultures of rumen methanogens.

Based on the PCoA analysis of sequence data, all the inhibitors and their combination, except PMSF and iodoacetamide, affected the overall microbiota. Interestingly, the three inhibitors and their three-way combination resulted in clear separation of the microbiota from the control, but the two-way combinations did not. The large variations among the replicates of imidazole plus PMSF and imidazole plus iodoacetamide are also difficult to explain.

Imidazole and the combinations containing imidazole increased the relative abundance of *Firmicutes*, a bacterial phylum containing primarily Gram-positive bacteria, which are more susceptible to lysozyme. One possible explanation is that
inhibition of the lysozyme protected members of *Firmicutes*, from digestion lysozyme produced by *E. caudatum* or other members of the cultures. This speculation is corroborated by the finding that consumption of lysozyme-rich milk decreased the relative abundance of *Firmicutes* in the feces of piglets (Maga et al., 2012). However, PMSF, an inhibitor of serine peptidase, also decreased the relative abundance of *Firmicutes*. The relative abundance of *Bacteroidetes*, the major Gram-negative phylum in the rumen and the *E. caudatum* cultures, showed the opposite trend as *Firmicutes*. It is not known if this is because of the increase in relative abundance of *Firmicutes*. The decrease of *Bacteroidetes* and the increase of *Firmicutes*, except in the presence of PMSF, could increase the ratio of *Firmicutes : Bacteroidetes*. Jami et al. (2014) demonstrated that *Firmicutes : Bacteroidetes* ratio was strongly positively correlated with daily milk fat yield in an *in-vivo* study. Therefore, the results suggest that supplementation of inhibitors except PMSF might have a potential to increase milk fat yield *in vivo*. *Proteobacteria* was represented in greater proportion in the *E. caudatum* cultures than in the rumen or *in vitro* rumen cultures. It is not known how this phylum became more adapted to the conditions of the *E. caudatum* cultures than those of the rumen. *Proteobacteria* was increased by imidazole and its combination with PMSF. Some genera of *Proteobacteria* such as genus *Desulfovibrio* show a correlation with milk-fat yield *in vivo* experiment (Jami et al., 2014). The increase of *Proteobacteria* might increase milk-fat yield, and real-time quantitative PCR method is needed to quantify some of the proteobacterial genera. Again, further *in vivo* experiment and real-time quantitative PCR method are needed to investigate if and how these inhibitors affect members of *Proteobacteria*. It should be cautioned that change in relative abundance of one phylum can change that of other phyla. More quantitative methods,
such as real-time phylum-specific PCR, are needed to accurately assess the effects of the inhibitors on the main bacterial phyla.

The distribution patterns of *Prevotella*, a Gram-negative genus of *Bacteroidetes*, and of *Streptococcus*, a Gram-positive genus of *Firmicutes*, resemble those of *Bacteroidetes* and *Firmicutes*, suggesting that the effects to those two phyla were primarily owning to the effects on these two genera. The increase in relative abundance of *Streptococcus* could be due to the decreased predation after *E. caudatum* was inhibited by the inhibitors. However, the lack of effect of PMSF on this genus does not support this premise. Previous studies have shown that *Streptococcus bovis* produces lactic acid, which reduces rumen pH (Otto, 1984). However, pH in this study didn’t show any decrease compared with the control. One probable explanation is that some lactating-using bacteria might have increased as well and increased consumption of lactic acid. Unfortunately, we did not analyze lactic acid concentration in the *E. caudatum* cultures. Quantitative analysis of *Streptococcus* using qPCR and lactic acid concentration is needed in future studies evaluating these inhibitors.

*Prevotella* plays significant roles in metabolism of proteins (Similarities, 2014). Although uncharacterized *Prevotella* sometimes predominate in the rumen (Firkins and Yu, 2015), the reduced relative abundance of *Prevotella* suggests that the supplementation of inhibitors might have affected rumen protein metabolism. *Prevotella* can degrade dietary protein and produce ammonia. It is not known if the decreased relative abundance of *Prevotella* also contributed to the decreased ammonia observed in the treatment cultures. *Butyrivibrio* species in rumen are major butyrate-producing bacteria and are involved in butyrate production (Similarities, 2014). The supplementation of inhibitors had mixed effects on the relative abundance of
Butyrivibrio, null effect from PMSF, iodoacetamide, and the three-way combination but decrease by imidazole and all the two-way combinations. Because the butyrate concentration was not significant changed, other butyrate-producing species were probably present and they might have compensated the decrease in Butyrivibrio by some of the inhibitors. Other minor genera, represented by less than 1% of the total sequences, were also affected by the inhibitors, differently for different inhibitors. Again, because changes in their relative abundance may not accurately reflect their actual changes, specific real-time PCR is needed to determine the effects of these inhibitors on these genera of bacteria that may be important to feed digestion and fermentation.
<table>
<thead>
<tr>
<th>Treatments</th>
<th>Control</th>
<th>Imi</th>
<th>PMSF</th>
<th>Iodo</th>
<th>Imi+PMSF</th>
<th>Imi+Iodo</th>
<th>PMSF+Iodo</th>
<th>Imi+PMSF+Iodo</th>
<th>SEM</th>
<th>p value</th>
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<td>24h</td>
<td>17030&lt;sup&gt;a&lt;/sup&gt;</td>
<td>3057&lt;sup&gt;c&lt;/sup&gt;</td>
<td>4585&lt;sup&gt;b&lt;/sup&gt;</td>
<td>2402&lt;sup&gt;cd&lt;/sup&gt;</td>
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<td>1965&lt;sup&gt;cd&lt;/sup&gt;</td>
<td>1747&lt;sup&gt;d&lt;/sup&gt;</td>
<td>1528&lt;sup&gt;d&lt;/sup&gt;</td>
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<td>1019&lt;sup&gt;bc&lt;/sup&gt;</td>
<td>2038&lt;sup&gt;b&lt;/sup&gt;</td>
<td>1019&lt;sup&gt;bc&lt;/sup&gt;</td>
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<td>1601&lt;sup&gt;b&lt;/sup&gt;</td>
<td>873&lt;sup&gt;bc&lt;/sup&gt;</td>
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<td>582&lt;sup&gt;bc&lt;/sup&gt;</td>
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</table>

Table 4.1 Effects of imidazole, PMSF, iodoacetamide and their combination on *E. caudatum* counts at 24, 48 and 72h *in vitro* incubation<sup>1</sup>

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<sup>1</sup> Count treatments x time interactions were insignificant (*p < 0.01*)

<sup>2</sup> Imi, imidazole at 100 mM; PMSF, phenylmethylsulphonyl fluoride at 3 mM; Iodo, iodoacetamide at 0.5 mM; Imi+PMSF, combination of 100 mM imidazole and 3 mM PMSF; Imi+Iodo, combination of 100 mM imidazole and 0.5 mM iodoacetamide; PMSF+Iodo, combination of 3 mM PMSF and 0.5mM iodoacetamide; Imi+PMSF+Iodo, combination of 100 mM imidazole, 3 mM PMSF, and 0.5 mM iodoacetamide.

<sup>3</sup> SEM, the standard error of the mean.

<sup>a-d</sup> Means followed by different superscripts in a row differ significantly (*p < 0.05*).
<table>
<thead>
<tr>
<th>Treatments 1</th>
<th>Control</th>
<th>Imi</th>
<th>PMSF</th>
<th>Iodo</th>
<th>Imi+PMSF</th>
<th>Imi+Iodo</th>
<th>PMSF+Iodo</th>
<th>Imi+PMSF+Iodo</th>
<th>SEM ³</th>
<th>p value</th>
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<td>6.71²</td>
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<td>NDF digestibility ²</td>
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<td>33.77</td>
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<td>34.12</td>
<td>33.41</td>
<td>34.82</td>
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<td>34.65</td>
<td>2.78</td>
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</table>

**Table 4.2** Effects of imidazole, PMSF, iodoacetamide and their combination on pH, NH3N concentration, DM digestibility, and neutral detergent fiber (NDF) digestibility in the *in vitro* fermentation.

¹ Imi, imidazole at 100 mM; PMSF, phenylmethylsulphonyl fluoride at 3 mM; Iodo, Iodoacetamide at 0.5 mM; Imi+PMSF, combination of 100 mM imidazole and 3 mM PMSF; Imi+Iodo, combination of 100 mM imidazole and 0.5 mM iodoacetamide; PMSF+Iodo, combination of 3 mM PMSF and 0.5 mM iodoacetamide; Imi+PMSF+Iodo, combination of 100 mM imidazole, 3 mM PMSF, and 3 mM iodoacetamide.

² pH, NH₃ concentration (mg N/L), and digestibility (%) of DM and NDF were determined at the end of 72 h *in vitro* incubation.

³ SEM, the standard error of the mean.

⁴ Means followed by different superscripts in a row differ significantly (p < 0.05).
Treatments\(^1\)  | Control | Imi | PMSF | Iodo | Imi+PMSF | Imi+Iodo | PMSF+Iodo | Imi+PMSF+Iodo | SEM\(^3\) | \(p\) value
--- | --- | --- | --- | --- | --- | --- | --- | --- | --- | ---
Acetate \(|\)  | 16.22 | 16.95 | 15.99 | 17.07 | 14.73 | 15.45 | 18.43 | 16.81 | 1.30 | 0.31
Propionate \(|\)  | 3.37 | 2.40 | 2.69 | 3.57 | 2.81 | 2.64 | 3.57 | 3.19 | 0.33 | 0.35
Isobutyrate \(|\)  | 0.56\(^c\) | 0.91\(^bc\) | 2.21\(^a\) | 1.90\(^a\) | 1.31\(^ab\) | 1.59\(^ab\) | 1.72\(^ab\) | 0.90\(^bc\) | 0.30 | <0.01
Butyrate \(|\)  | 1.67 | 1.27 | 1.44 | 1.61 | 1.50 | 1.72 | 1.01 | 1.20 | 0.25 | 0.46
Isovalerate \(|\)  | 0.40 | 0.88 | 0.49 | 1.27 | 1.80 | 1.05 | 0.81 | 1.40 | 0.21 | 0.09
Valerate \(|\)  | 0.25 | 0.20 | 0.27 | 0.41 | 0.40 | 0.61 | 0.42 | 0.78 | 0.15 | 0.18
Total VFA \(|\)  | 22.48 | 22.62 | 23.09 | 25.83 | 22.54 | 23.07 | 25.96 | 24.29 | 4.55 | 0.54
A:P \(^2\) \(|\)  | 4.81 | 7.06 | 5.94 | 4.78 | 5.24 | 5.85 | 5.16 | 5.27 | 0.67 | 0.07

| Table 4.3 | Effect of imidazole, PMSF, iodoacetamide and their combinations on volatile fatty acids (VFA) concentrations (mM) in the \(in vitro\) \(E. caudatum\) cultures. |

\(^1\) Imi, imidazole at 100 mM; PMSF, phenylmethylsulphonyl fluoride at 3 mM; Iodo, Iodoacetamide at 0.5 mM; Imi+PMSF, combination of 100 mM imidazole and 3 mM PMSF; Imi+Iodo, combination of 100 mM imidazole and 0.5 mM iodoacetamide; PMSF+Iodo, combination of 3 mM PMSF and 0.5 mM iodoacetamide; Imi+PMSF+Iodo, combination of 100 mM imidazole, 3 mM PMSF, and 3 mM iodoacetamide.

(Continued)
(Table 4.3 Continued)

2 A : P, acetate to propionate ratio.

3 SEM, the standard error of the mean.

\[^{a-c}\text{Means followed by different superscripts in a row differ significantly (}\text{p} < 0.05\).\]
Table 4.4 Effect of imidazole, PMSF, iodoacetamide, and their combinations on relative abundance of bacteria and archaea in the *in vitro* *E. caudatum* cultures when treated with the inhibitors for 72h.

1 Imi, imidazole at 100 mM; PMSF, phenylmethylsulphonyl fluoride at 3 mM; Iodo, Iodoacetamide at 0.5 mM; Imi+PMSF, combination of 100 mM imidazole and 3 mM PMSF; Imi+Iodo, combination of 100 mM imidazole and 0.5 mM iodoacetamide; PMSF+Iodo, combination of 3 mM PMSF and 0.5 mM iodoacetamide; IPI, combination of 100 mM imidazole, 3 mM PMSF, and 3 mM iodoacetamide.

2 SEM, the standard error of the mean.

*a-c* Means followed by different superscripts in a row differ significantly (*p* < 0.05).
<table>
<thead>
<tr>
<th>Phylum</th>
<th>Control</th>
<th>Imi</th>
<th>PMSF</th>
<th>Iodo</th>
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<th>IPI</th>
<th>SEM²</th>
<th>p value</th>
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</thead>
<tbody>
<tr>
<td>Bacteroidetes</td>
<td>47.54</td>
<td>5.73</td>
<td>46.20</td>
<td>28.16</td>
<td>4.60</td>
<td>7.11</td>
<td>6.08</td>
<td>23.03</td>
<td>1.18</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>Proteobacteria</td>
<td>37.78</td>
<td>55.87</td>
<td>36.11</td>
<td>32.40</td>
<td>56.74</td>
<td>30.55</td>
<td>18.80</td>
<td>32.88</td>
<td>2.12</td>
<td>0.01</td>
</tr>
<tr>
<td>Firmicutes</td>
<td>8.17abcd</td>
<td>34.11abcd</td>
<td>14.24</td>
<td>40.81</td>
<td>35.71abcd</td>
<td>59.82abcd</td>
<td>72.97abcd</td>
<td>41.77</td>
<td>2.71</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>Synergistetes</td>
<td>2.89a</td>
<td>2.73a</td>
<td>0.63</td>
<td>0.86</td>
<td>1.63</td>
<td>1.25</td>
<td>0.95</td>
<td>0.67</td>
<td>0.10</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>Verrucomicrobia</td>
<td>0.82a</td>
<td>0.33b</td>
<td>0.07</td>
<td>0.18</td>
<td>0.24</td>
<td>0.26</td>
<td>0.33</td>
<td>0.13</td>
<td>0.03</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>Fibrobacteres</td>
<td>0.87b</td>
<td>0.10b</td>
<td>0.81</td>
<td>0.20</td>
<td>0.00</td>
<td>0.00</td>
<td>0.00</td>
<td>0.06</td>
<td>0.02</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>Spirochaetes</td>
<td>0.47b</td>
<td>0.00b</td>
<td>0.81</td>
<td>1.63</td>
<td>1.25</td>
<td>0.95</td>
<td>0.67</td>
<td>0.10</td>
<td>&lt;0.01</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>Chloroflexi</td>
<td>0.21a</td>
<td>0.06b</td>
<td>0.10</td>
<td>0.11</td>
<td>0.08</td>
<td>0.11</td>
<td>0.09</td>
<td>0.03</td>
<td>0.01</td>
<td>0.17</td>
</tr>
<tr>
<td>Lentisphaerae</td>
<td>0.14a</td>
<td>0.03bc</td>
<td>0.02</td>
<td>0.05</td>
<td>0.04</td>
<td>0.08</td>
<td>0.07bc</td>
<td>0.06bc</td>
<td>0.01</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>Planctomycetes</td>
<td>0.06c</td>
<td>0.01b</td>
<td>0.03</td>
<td>0.02</td>
<td>0.01</td>
<td>0.01</td>
<td>0.01</td>
<td>0.01</td>
<td>0.01</td>
<td>0.02</td>
</tr>
<tr>
<td>Elusimicrobia</td>
<td>0.01c</td>
<td>0.01a</td>
<td>0.00</td>
<td>0.01</td>
<td>0.01</td>
<td>0.01</td>
<td>0.01</td>
<td>0.01</td>
<td>&lt;0.01</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>Actinobacteria</td>
<td>0.01a</td>
<td>0.04a</td>
<td>0.05</td>
<td>0.00</td>
<td>0.01</td>
<td>0.01</td>
<td>0.01</td>
<td>0.01</td>
<td>0.01</td>
<td>0.18</td>
</tr>
<tr>
<td>Cyanobacteria</td>
<td>0.00a</td>
<td>0.03a</td>
<td>0.00</td>
<td>0.01</td>
<td>0.04</td>
<td>0.04</td>
<td>0.03</td>
<td>0.05</td>
<td>0.01</td>
<td>0.62</td>
</tr>
<tr>
<td>Euryarchaeota</td>
<td>0.78a</td>
<td>0.56ab</td>
<td>0.14</td>
<td>0.13</td>
<td>0.48</td>
<td>0.30</td>
<td>0.26</td>
<td>0.28</td>
<td>0.04</td>
<td>0.02</td>
</tr>
</tbody>
</table>

Table 4.5 Effect of imidazole, PMSF, iodoacetamide, and their combinations on relative abundance of bacterial phyla in the *in vitro* *E. caudatum* cultures when treated with the inhibitors for 72h.¹

¹ Imi, imidazole at 100 mM; PMSF, phenylmethylsulphonyl fluoride at 3 mM; Iodo, iodoacetamide at 0.5 mM; Imi+PMSF, combination of 100 mM imidazole and 3 mM PMSF; Imi+Iodo, combination of 100 mM imidazole and 0.5 mM iodoacetamide; PMSF+Iodo, (Continued)
(Table 4.5 Continued)

combination of 3 mM PMSF and 0.5 mM iodoacetamide; IPI, combination of 100 mM imidazole, 3 mM PMSF, and 3 mM iodoacetamide.

2 SEM, the standard error of the mean.

\textsuperscript{a-d} Means followed by different superscripts in a row differ significantly ($p < 0.05$)
### Table 4.6 Effect of imidazole, PMSF, iodoacetamide, and their combinations on relative abundance of bacterial genera in the *in vitro* *E. caudatum* cultures when treated with the inhibitors for 72h\(^1\).

<table>
<thead>
<tr>
<th>Genus</th>
<th>Control</th>
<th>Imi</th>
<th>PMSF</th>
<th>Iodo</th>
<th>Imi+PMSF</th>
<th>Imi+Iodo</th>
<th>PMSF+Iodo</th>
<th>IPI</th>
<th>SEM(^2)</th>
<th>(p) value</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Prevotella</em></td>
<td>34.66(^a)</td>
<td>1.18(^c)</td>
<td>38.50(^a)</td>
<td>23.32(^c)</td>
<td>2.06(^c)</td>
<td>3.50(^c)</td>
<td>2.06(^c)</td>
<td>19.40(^b)</td>
<td>1.09</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td><em>Streptococcus</em></td>
<td>0.09(^d)</td>
<td>25.07(^c)</td>
<td>0.69(^d)</td>
<td>35.57(^bc)</td>
<td>31.24(^bc)</td>
<td>56.00(^ab)</td>
<td>69.53(^a)</td>
<td>37.93(^bc)</td>
<td>2.71</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td><em>Butyrivibrio</em></td>
<td>0.16(^a)</td>
<td>0.05(^b)</td>
<td>0.30(^a)</td>
<td>0.22(^a)</td>
<td>0.05(^b)</td>
<td>0.08(^b)</td>
<td>0.06(^b)</td>
<td>0.20(^a)</td>
<td>0.02</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td><em>Ruminococcus</em></td>
<td>0.64(^a)</td>
<td>0.33(^a)</td>
<td>3.06(^a)</td>
<td>0.62(^b)</td>
<td>0.77(^b)</td>
<td>0.76(^b)</td>
<td>0.38(^b)</td>
<td>0.36(^b)</td>
<td>0.18</td>
<td>0.05</td>
</tr>
<tr>
<td>gL7AE11</td>
<td>0.00(^b)</td>
<td>1.96(^a)</td>
<td>0.00(^b)</td>
<td>0.00(^b)</td>
<td>0.00(^b)</td>
<td>0.01(^b)</td>
<td>0.00(^b)</td>
<td>0.00(^b)</td>
<td>0.07</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td><em>p-75-a5</em></td>
<td>0.02(^b)</td>
<td>2.17(^a)</td>
<td>0.00(^b)</td>
<td>0.02(^b)</td>
<td>0.03(^b)</td>
<td>0.03(^b)</td>
<td>0.07(^b)</td>
<td>0.00</td>
<td>0.09</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td><em>Treponema</em></td>
<td>0.11(^b)</td>
<td>0.05(^b)</td>
<td>0.84(^a)</td>
<td>0.17(^b)</td>
<td>0.05(^b)</td>
<td>0.06(^b)</td>
<td>0.09(^b)</td>
<td>0.11(^b)</td>
<td>0.03</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td><em>Pyramidobacter</em></td>
<td>1.31(^a)</td>
<td>0.32(^b)</td>
<td>0.15(^b)</td>
<td>0.25(^b)</td>
<td>0.22(^b)</td>
<td>0.28(^b)</td>
<td>0.39(^b)</td>
<td>0.18(^b)</td>
<td>0.04</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td><em>Fibrobacter</em></td>
<td>0.47(^c)</td>
<td>0.00(^c)</td>
<td>0.81(^a)</td>
<td>0.20(^c)</td>
<td>0.00(^c)</td>
<td>0.00(^c)</td>
<td>0.00(^c)</td>
<td>0.06(^c)</td>
<td>0.02</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td><em>Ruminobacter</em></td>
<td>21.44(^ab)</td>
<td>8.94(^abcd)</td>
<td>7.71(^bcd)</td>
<td>19.23(^abc)</td>
<td>11.06(^abcd)</td>
<td>5.10(^cd)</td>
<td>3.00(^d)</td>
<td>23.57(^a)</td>
<td>1.35</td>
<td>0.04</td>
</tr>
<tr>
<td><em>Succinivibrio</em></td>
<td>0.18(^b)</td>
<td>3.38(^a)</td>
<td>0.31(^b)</td>
<td>0.14(^b)</td>
<td>3.02(^ab)</td>
<td>0.72(^ab)</td>
<td>0.06(^b)</td>
<td>2.01(^ab)</td>
<td>0.27</td>
<td>0.05</td>
</tr>
</tbody>
</table>

\(^1\)Imi, imidazole at 100 mM; PMSF, phenylmethylsulphonyl fluoride at 3 mM; Iodo, Iodoacetamide at 0.5 mM; Imi+PMSF, combination of 100 mM imidazole and 3 mM PMSF; Imi+Iodo, combination of 100 mM imidazole and 0.5 mM iodoacetamide; PMSF+Iodo,
(Table 4.6 Continued)

combination of 3 mM PMSF and 0.5 mM iodoacetamide; IPI, combination of 100 mM imidazole, 3 mM PMSF, and 3 mM iodoacetamide.

SEM, the standard error of the mean.

Means followed by different superscripts in a row differ significantly ($p < 0.05$).
Figure 4.1 Principal coordinate analysis of bacterial communities of the *in vitro* *E. caudatum* cultures after treatment with the inhibitors for 72 h. C, control; Imi, imidazole at 100 mM, PMSF, phenylmethylsulphonyl fluoride at 3 mM; Iodo, iodoacetamide at 0.5 mM; Imi+PMSF, combination of 100 mM imidazole and 3 mM PMSF; Imi+Iodo, combination of 100 mM imidazole and 0.5 mM iodoacetamide; PMSF+Iodo, combination of 3 mM PMSF and 0.5 mM iodoacetamide; Imi+PMSF+Iodo, combination of 100 mM imidazole, 3 mM PMSF, and 3 mM iodoacetamide.
Figure 4.2 Relative abundance (represented by % of total sequences) of the major bacterial phyla identified in the \textit{in vitro} \textit{E. caudatum} cultures.

Imi, imidazole at 100 mM; PMSF, phenylmethylsulphonyl fluoride at 3 mM; Iodo, Iodoacetamide at 0.5 mM; Imi+PMSF, combination of 100 mM imidazole and 3 mM PMSF; Imi+Iodo, combination of 100 mM imidazole and 0.5 mM iodoacetamide; PMSF+Iodo, combination of 3 mM PMSF and 0.5 mM iodoacetamide; IPI, combination of 100 mM imidazole, 3 mM PMSF, and 3 mM iodoacetamide.
Chapter 5: Conclusion

All the inhibitors of lysozyme and peptidases tested substantially inhibited *E caudatum*, the most dominant species of rumen protozoa. Evidently, *E. caudatum* depends on these digestive enzymes to digest the engulfed bacteria for essential nutrients. The concurrent decrease in ammonia concentration suggests that these inhibitors decreased degradation of proteins, most likely microbial protein, and or ammonia genesis. Because they did not adversely affect the digestibility of DM and NDF or fermentation characteristics, these inhibitors may hold potential to be used to control rumen protozoa and improve nitrogen utilization efficiency. However, these inhibitors affected the microbiota by decreasing the phylum *Bacteroidetes* and its genus *Prevotella* and increasing the phylum *Firmicutes* including its genus *Streptococcus*. Further research using quantitative analysis such as real-time PCR is needed to determine the effect on individual genera and phyla of bacteria. The *E. caudatum* cultures used in this study appeared to have very different microbiota than that of the rumen. Studies using *in vitro* rumen cultures inoculated from fresh rumen fluid should also be conducted to assess the effect of these inhibitors on the rumen microbes typically present in the rumen, including other protozoa. Of course, *in vivo* experiments are needed to further verify these inhibitors, but toxicity information needs to be obtained first, either from the literature or from animal experiments. Furthermore, the
E. caudatum culture used in this study had not been exposed to the inhibitors before. Thus, research using long-term exposure to these inhibitors is also needed to examine if E. caudatum can become adapted to the inhibitors.
References


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Bhatia, I. S. (1980). Characterization of Enzymes of Ammonia Metabolism in Protozoa from Rumen of Buffalo Bulls BY AJAIB SINGH ’.., half of the microbial protein in the reticulo rumen (4). Studies were conducted to characterize the enzymes of ammonia metabolism in rumen protozoa in the buffalo as this aspect appears to have been little investigated. The effect of ATP concentrations on the assimilation of NH, as glutamine was also, 371, 364–371.


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https://doi.org/10.1371/journal.pone.0085423


https://doi.org/10.1016/j.anifeedsci.2005.08.004


Increases the Abundance of Cellulolytic Ruminococci and Methanogens but does not Affect the Bacterial and Methanogen Diversity in the Rumen of Sheep. *J Anim Sci*, 89(3), 783-91


