ANTONIO PINHEIRO FACIOLA

Effects of Lauric Acid on Fermentation Patterns, Ruminal Protozoa, and Performance of Dairy Cows

Thesis submitted to the Federal University of Viçosa in partial fulfillment of the requirements of the Graduate Program in Animal Science for the degree of "*Magister Scientiae*".

VIÇOSA
MINAS GERAIS – BRAZIL
2004

Ficha catalográfica preparada pela Seção de Catalogação e Classificação da Biblioteca Central da UFV

T
F141e
2004

Faciola, Antonio Pinheiro, 1979-
Effects of lauric acid on fermentation patterns, ruminal protozoa, and performance of dairy cows / Antonio Pinheiro Faciola. – Viçosa : UFV, 2004.
xvi, 102f. : il. ; 29cm.

Resumo em português.

Orientador: Maria Ignez Leão
Dissertação (mestrado) - Universidade Federal de Viçosa.

Inclui bibliografia.

1. Ruminante - Nutrição. 2. Rúmen - Fermentação - Efeito dos ácidos graxos. 3. Ácido láurico. 4. Bovino de leite - Parasito. 5. Bovino de leite - Desempenho. I. Universidade Federal de Viçosa. II.Título.

CDD 20.ed. 636.20852

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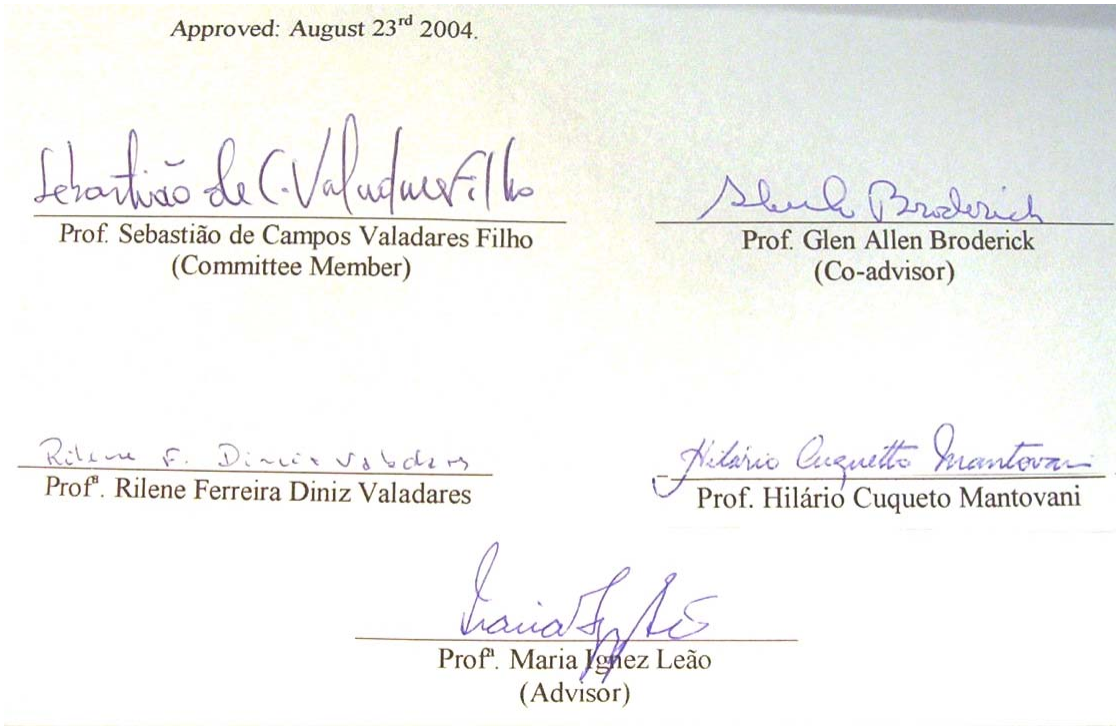
Approved: August 23rd 2004.



DEDICATORY

I dedicate this thesis to the memory of my beloved dad, João Eduardo Cardoso Faciola, whom could not share this and my many other achievements in my life personally, but will be always with me in my heart and thoughts: And to my wonderful mom, Regina Pinheiro Faciola, the most important person in my life, who made me what I am, sacrificing everything for my own good. Words would never be enough to express my love for her. I wish that some day I could approach such a level of love in raising a family.

ACKNOWLEDGMENTS

First of all, I would like to thank My Lord God who kept me healthy and full of faith to accomplish all my goals.

I am thankful to the Federal University of Viçosa for their excellence in teaching, researching, and extension, which provided me a singular education.

I am also thankful to the U. S. Dairy Forage Research Center, which allowed me to conduct the experimental phase of my study and provided financial support during my stay in the United States.

I would like to thank CAPES for the assistantship provided during my graduate program, and to CNPq for the assistantship provided during my undergraduate program, which first opened my eyes to research.

The research conducted during my Masters of Science program would not have been done without the assistance, friendship, and love of a great number of people.

I am thankful to the love and constant prayers of my family (my great-grandmother Mãe-de-Mira, my grandmother Pastora, my aunts Léa, Célia, Telma, Maria, and Conceição; my uncles Otávio, Reginaldo, Fernando, Jorge and Sam, and all of my cousins).

To the immense help and care of my sister Gina, and my brother-in-law John, and my lovely nephews Nolan, Taylor, and niece Daniela.

To my advisor Dr. Maria Ignez Leão. She is an example of dedication and determination; a woman who is a milestone in Animal and Veterinary Science in Brazil; moreover, a great person, easygoing, full of humor, whom I always had a pleasure being with. Ignez gave me all the support and advising I needed throughout my Master's. There is no one else in the milieu whom I would have rather worked for. I will always be pleased to say that I did my Master's work under Ignez's supervision.

To Dr. Glen Broderick, whom I wish to express my most sincere appreciation for the unique opportunity to come to the USA and for the warm reception. Dr. Glen is a wonderful human-being, generous, patient, extremely polite, humble, humorous, and intelligent. An example of a scientist and man. His guidance in planning and executing these experiments, thoughtful discussions, and support throughout my research were greatly appreciated. I am honored by the chance of working with such a distinguished man.

To Dr. Alexander Hristov and the members of my thesis' committee, Professors Sebastião Valadares, Hilário Mantovani, and Rilene Valadares for the thoughtful comments.

To all my professors at the UFV, especially those whom I spent a lot of time with; Marcelo Rodrigues, Théa Machado, Mario Paulino and Odilon Pereira.

To Jill Davidson and the barn crew, for feeding and animal care at the USDFRC Research Farm (Prairie du Sac, WI).

To Mary Becker, Wendy Radloff, Fern Kanitz, Veronica Gaertner, Adam Ford, Daniel Diederich, John Mackenzie, Carla Bearzi and Marcos Marcondes for assisting with sampling and laboratory analyses.

To Santiago Reynal, Jesus Olmos and Andre Brito for the thoughtful comments and help.

To my “brothers” Fred, Wil, and Vinícius; whom shared with me the best years of my life teaching me so many things that would not fit in this thesis. Nothing can define the real meaning of a true friendship.

To my great friends that I made in Viçosa, BH, Rio, and Belém, so many that is hard mention them all. Mario and Fê Chizzottis, Cris and Ké, Jane, George Japa, Leidi, Mônica, Rafael, Renan, Cristiano, Léo, Fabrício, Tigrão, Valquíria, Pat, Polianinha, Polyana, Joana, Ricardos (Pará, Nariz, and Silvio), Dudu, Boteco, Karla and Pedro, Américo, Silas, Gaúcho, Marco and Érika, Carla, Renata, Sérgio, André, Daniela, Márcia, Alex, Giselly, Viviane, Marco Túlio, Paulo, Caike, and Antônio Arthur.

To the unforgettable “Zoo-98”

To all my Brazilians fellows in Madison whom make this city warmer, especially Aldo, Alex, Andrea, André, Carla, Carol, Daniel, Fê, Gabriel, Helena, Letícia, Luciano, Marcel, Márcio, Mariana, Jerônimo, and Tito.

To all my good friends at Babcock House and my friends from the states and overseas.

BIOGRAPHY

ANTONIO PINHEIRO FACIOLA, son of João Eduardo Cardoso Faciola and Regina de Nazaré Pinheiro Faciola, was born in Rio de Janeiro, RJ, Brazil on July 24th 1979.

He pursued his bachelor degree in Animal Science at the Federal University of Viçosa and graduated in September 2002.

From September 1999 to September 2002 he had a scholarship from CNPq to work in the Laboratory of Ruminant Nutrition and at the Dairy Goat facilities under the supervision of Dr. Marcelo Rodrigues.

In September 2002, he started his graduate studies at the Animal Science Department from the same University, focusing his research in Ruminat Nutrition under supervision of Dr. Maria Ignez Leão.

In September 2003, he went to the United States of America to conduct experiments at the Dairy Forage Research Center facilities in the state of Wisconsin, under the supervision of Dr. Glen Broderick.

In July 2004, he was admitted by The Graduate School of The University of Wisconsin – Madison into the PhD program in the Dairy Science Department.

He submitted his Masters' thesis to the committee in August 2004.

"It was thus that science was constructed: not by the prudence of who march, but by the dares of who dream. All knowledge starts with a dream. The knowledge is nothing else but an adventure through the unknown sea; seek the dreamed land. However, to dream is something that is not taught. It sprouts from deep inside the body, like the water sprouts from deep inside of the Land." (Rubem Alves).

"Foi assim que se construiu a ciência: não pela prudência dos que marcham, mas pela ousadia dos que sonham. Todo conhecimento começa com um sonho. O conhecimento nada mais é que a aventura pelo mar desconhecido, em busca da terra sonhada. Mas sonhar é coisa que não se ensina. Brota das profundezas do corpo, como a água brota das profundezas da Terra." (Rubem Alves).

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RESUMO

FACIOLA, Antonio Pinheiro, M.S., Universidade Federal de Viçosa, Agosto de 2004. **Efeito de Ácido Láurico sobre a Fermentação Ruminal, População de Protozoários no Rúmen e Desempenho de Vacas Leiteiras**. Orientadora: Maria Ignez Leão. Co-orientador: Glen Allen Broderick. Conselheiro: Sebastião de Campos Valadares Filho.

Os protozoários ruminais exercem efeito negativo sobre a utilização de proteína em ruminantes, reduzindo a proteína de origem microbiana sintetizada no rúmen e a PNDR, que são as principais fontes da AA para os ruminantes. Dessa forma, os objetivos deste trabalho foram: (1) Avaliar a viabilidade do uso do Ácido Láurico (C12:0) como um defaunador de rotina e (2) avaliar os efeitos da defaunação parcial do rúmen sobre a fermentação ruminal e o desempenho de vacas leiteiras. No primeiro experimento, seis vacas da raça Holandesa (sendo uma seca), com media de 660 (SD 46) kg de PV; 3,5 (SD 1) partos; 102 (SD 23) dias de lactação e 40,4 (SD 6,2) kg/leite/dia; canuladas no rúmen foram divididas com base nos dias em lactação em 2 grupos de três, em um delineamento inteiramente casualisado. Os tratamentos foram: 1) Controle, 2) 160 g/d de AL e 3) 160 g/d de laurato de sódio. Ambos AL e LANa foram

infundidos em dose única via canula ruminal diariamente antes da alimentação. A ração total continha (na base da MS): 15% de silagem de alfafa, 40% de silagem de milho, 30% de silagem de milho úmido, 14% de farelo de soja, 1% de premix de vit. e minerais, tendo a dieta total, 16,6% PB e 29% FDN. No Segundo experimento, 32 vacas da raça Holandesa multíparas (8 canuladas no rúmen) com média de 633 (SD 52) kg de PV; 152 (SD 72) dias em lactação; 2,6 (SD 1) partos e 43,1 (SD 11) kg/leite/dia e 20 vacas da raça Holandesa primíparas com media de 566 (SD 39) kg de PV; 123 (SD 42) dias em lactação e 40,5 (SD 2,6) kg/leite/dia foram divididas com base nos dias em lactação em grupos de 4, formando 8 blocos de multíparas (2 com vacas canuladas) e 5 blocos de primíparas em um delineamento interamente casualisado. Antes do início dos tratamentos, todas os animais foram alimentados com a mesma dieta (controle) por 2 semanas em um período de co-variância, onde a produção de leite e a contagem de protozoários foram determinadas para utilização nas análises estatísticas. Os animais dentro dos blocos foram então aleatóriamente distribuídos em um dos quatro tratamentos, onde foram alimentadas por 8 semanas. As quatro dietas experimentais foram semelhantes, onde parte do milho moído foi substituído por um premix constituído de 8% de AL e 92% de milho moído. O premix com AL foi adicionado nas proporções de 0; 4,05; 8,11 e 12,16% na base da MS da dieta (0, 80, 160 e 240g/LA/d). Todos os animais foram injetados com bST a cada 14 dias. Os dados foram analisados usando o procedimento Proc Mixed do programa estatístico SAS. Ambos AL e LANa apresentaram efeito altamente inibitórios aos protozoários ruminais quando dosados em 160 g/d via canula ruminal, reduzindo a população de protozoários em 90%. AL reduziu a concentração de amônia ruminal em 60% sem reduzir o CMS. Ambos agentes reduziram a concentração de aminoácidos livres no rúmen. AL não afetou o pH ruminal, nem reduziu a concentração total de AGV no rúmen. Nas condições do primeiro

experimento, AL se mostrou um potente agente dafaunador, evitando a necessidade de utilizar LANa que é mais caro. AL fornecido na dieta em níveis de 80, 160 e 240 g/d não reduziu o CMS, não afetou o pH ruminal, os parâmetros fermentativos no rúmen e a produção de leite. AL misturado na dieta total de vacas da raça Holandesa, em níveis de 160 e 240 g/d reduziu a população de protozoários ruminais em apenas 25 e 30%, respectivamente, mostrando que estes níveis de AL na dieta não foram suficientes para permitir atingir uma concentração no rúmen que fosse suficiente para promover os efeitos defaunadores do AL.

ABSTRACT

FACIOLA, Antonio Pinheiro, M.S., Universidade Federal de Viçosa, August 2004. **Effects of Lauric Acid on Fermentation Patterns, Ruminal Protozoa, and Performance of Dairy Cows.** Advisor: Maria Ignez Leão. Co-advisor: Glen Allen Broderick. Committee Member: Sebastião de Campos Valadares Filho.

Ruminal ciliate protozoa have a negative effect on protein utilization in ruminants by reducing both microbial protein and RUP flow out of the rumen. The aims of this study were: (1) To evaluate lauric acid as a practical defaunating agent, (2) to assess the effects of partial defaunation on fermentation patterns and milk production in dairy cows. In the first trial, six multiparous Holstein cows (one dry), averaging 660 (SD 46) kg of BW, parity 3.5 (SD 1), 102 (SD 23) DIM, and 40.4 (SD 6.2) kg/d of milk; fitted with ruminal cannulae were blocked into groups of 3 by DIM to give 2 blocks in a trial of randomized complete block design. The treatments were: 1) Control, 2) 160g/d of LA, or 3) 160g/d of sodium laurate. Both LA and NaLA were given in a single dose into the rumen via cannulae before feeding. The TMR contained (DM basis): 15% alfalfa silage, 40% corn silage, 30% rolled high moisture corn, 14% soybean meal, 1% Vit. & Min. premix, 16.6% CP and 29% NDF. In the second trial, thirty-two multiparous Holstein cows (8 with ruminal cannulae) averaging parity 2.6 (SD 1), 633 (SD 52) kg of

BW, 152 (SD 72) DIM, and 43.1 (SD 11) kg milk/d, and twenty primiparous Holstein cows averaging 566 (SD 39) kg of BW, 123 (SD 42) DIM, and 40.5 (SD 2.6) kg milk/d were blocked into groups of 4 by DIM to give 8 multiparous blocks (2 cows with ruminal cannulae) and 5 primiparous blocks in a trial of randomized complete block design. Prior to starting the experimental phase of the trial, all cows were fed the same diet (control) for a 2-wk covariate period and production of milk and protozoal counts were determined for use in statistical analysis. Cows within blocks of 4 were then randomly assigned to one of the 4 diets and fed only that diet during the remaining 8 weeks of the study. The four experimental diets were similar, except that some of the finely ground dry corn was replaced with finely ground dry corn plus lauric acid (8% LA and 92% corn, DM basis) in stepwise increments from 0 to 12.16% of dietary DM. All cows were injected biweekly with bST. Data were analyzed using the Proc Mixed procedure of SAS. Both lauric acid and sodium laurate showed high anti-protozoal activity when dosed at 160 g/d via ruminal canulae, reducing the ciliate population within the rumen by 90%. Lauric acid reduced ruminal ammonia concentration by 60% without reducing DMI. Both agents reduced ruminal free amino acid concentration. Lauric acid did not affect ruminal pH, nor reduce total ruminal VFA concentration. Under the condition of the first trial, Lauric Acid was shown to be a potent defaunation agent, obviating the need to use Na-laurate, which is more costly. Lauric acid fed on the diets at 80, 160, and 240 g/d did not reduce DMI, did not affect ruminal pH, ruminal parameters, or milk production. Lauric acid fed at 160 and 240 g/d on the TMR of Holstein's dairy cows reduced ruminal protozoal population by only 25 and 30% respectively, showing that these levels on the diet were not sufficient to achieve a concentration within the rumen that promote the anti-protozoal effect of lauric acid.

CHAPTER 1

LITERATURE REVIEW

1.1 Introduction

The ciliate protozoa were the first ruminal microorganisms to be described (Orpin 1984). Reported for the first time by Gruby and Delafond (1843), ruminal protozoa have been the subject of many studies in ruminant nutrition. Although they have been shown to be nonessential for the host animal (Becker et al. 1929), their role in ruminal ecology, fermentation, animal health and performance remains debatable.

Although they can comprise as much as 50% of the total microbial mass in the rumen, protozoa generally provide only 20% of the total microbial N entering the duodenum (Jouany et al. 1988). This is probably due to the selective retention of protozoa in the rumen (Weller and Pilgrim 1974; Jouany and Senaud 1978; Harrison et al. 1979; Coleman et al. 1980; Leng et al. 1981; John and Ulyatt 1984; Punia and Leibholz 1984; and Michalowski et al. 1986).

Populations considered normal across most rations for domestic ruminants are concentrations of about of 1 x 10^6 cells per ml of ruminal contents. The largest, most obvious and most important protozoa are the ciliates, of which there are two groups: the

holotrich and the entodiniomorphid. This is the reason why ruminal protozoa commonly are referred to as ciliates.

Probably the best known effect of ruminal protozoa is their ability to degrade insoluble protein. They are able to ferment feed protein and microbial protein, the two main sources of amino acids used by ruminant animals.

The role of protozoa on recycling bacterial N has been studied (Aharoni et al. 1991; Firkins et al. 1992; and Koenig et al. 2000) and it has been reported that a single protozoa can uptake $10^2 – 10^4$ bacteria/h (Coleman 1975), which could represent a major loss in protein to the animal host.

Reduction on protozoa population has been associated with lower ruminal ammonia concentration and higher flow of ruminal undegradable protein and microbial protein (Jouany 1996) and amino acids (Veira et al. 1984) to the duodenum.

It is widely accepted that protozoa slow the fermentation of readily fermentable carbohydrates in the rumen, limiting the decrease in pH and lactic acid production. Most in vivo studies have shown that defaunation lowers cell-wall carbohydrate digestion throughout the digestive tract by as much as 5-15% (Jouany et al. 1988). However, apparent starch digestion in the rumen is generally not affected in vivo by defaunation (Ushida et al. 1991). Defaunation also increases the molar proportion of propionate, decreases the molar proportion of acetate and butyrate, and has a slight negative effect on total VFA production (Veira 1986; Jouany et al. 1988; and Ushida et al. 1991).

Other effects of defaunation are: reduction on methane formation in the rumen (Czerkawski et al. 1966; Newbold et al. 1995; Ushida and Jouany 1996; Dong et al. 1997; Dohme et al. 1999; Machmuller and Kreuzer 1999; Machmuller et al. 2001; Machmuller et al. 2002; Machmuller et al. 2003b; and Machmuller et al. 2003a), increasing the flow

of unsaturated fatty acids (C18:2 and C18:3) from the rumen (Hsu et al. 1991b), and enhancement the copper bioavailability (Ivan et al. 1986).

The performance of protozoa-free animals is related to the defaunation method. Moreover, the conditions (diet, physiological status, production level) in which total or partial defaunation can be recommended to improve the efficiency of nutrient utilization by animals is still uncertain; it was proposed in the late 1970's that diets high in energy and low in protein, given to animals that have high requirements for amino acids, could have positive effects on performance.

We have to keep in mind that the ciliates are an integral part of the ruminant system where relationships such as predation, competition for nutrients, and synergism exists between protozoa and the other microorganisms. Furthermore, defaunation does not mean merely the elimination of protozoa from the rumen, but also the loss of ecological interrelationships that affect the size, generic distribution, and metabolic activity of bacteria and fungal populations.

1.2 Effects of Protozoa on Nitrogen Metabolism

1.2.1 Degradation of Dietary Proteins

Entodinomorphid protozoa are particularly efficient in taking up particulate matter suspended in ruminal fluid (Jouany 1996). This group also has shown low uptake and a limited transport of soluble substances across the cell membrane (Coleman 1986b). In contrast, holotrichs seem to play a major role in utilizing soluble nutrients (Williams and Coleman 1992) instead of insoluble particles.

All species of entodiniomorphids possess an extrudable U-shaped peristome on which is a band of cilia that runs round the outside and then down one side of the U-shaped oesophagus to the cytostome (Coleman and Hall 1971). They use the cilia to trap

particles that are subsequently driven into a vestibulum and then into the cystostome (Jouany 1996).

Most protozoa assimilate free amino acids by active or passive transport and incorporate them directly into their proteins (Chesters 1968; and Hoshino and Sugiyama 1974). Unlike bacteria, protozoa do not have urease (Onodera et al. 1977) and cannot use urea or ammonia to synthesize amino acids.

Transamination and decarboxylation of amino acids in protozoa have also been reported (Coleman 1967; and Tsubota and Hoshino 1969). Metabolic pathways for lysine, arginine, threonine, tryptophan, and methionine in protozoa were identified (Onodera et al. 1997). Of those pathways, decarboxylation of diaminopimelic acid to lysine would seem to be important in ruminant nutrition if lysine is limited in the diet, because diaminopimelic acid, a component of the bacterial cell wall, is not utilized by the host (Mason and White 1971) unless first converted to lysine by protozoa. Amino acid uptake increases with increasing amino acid concentration (Williams and Coleman 1992). The same authors also describe the metabolism of different amino acids by ciliates. As pointed out by Williams and Coleman (1992), all experiments on the uptake of free amino acids by rumen ciliates must consider that these microbes harbor bacteria in vesicles in the endoplasm and attached to the outside membrane. Thus, it is possible for free amino acids to be taken up by bacteria, which are then engulfed by protozoa.

Active protozoal proteases have been found in several studies (Abou Akkada and Howard 1962; Gutierrez and Davis 1962; Naga and El-Shazly 1968; Onodera and Kandatsu 1970; Coleman 1983; Forsberg et al. 1984; and Nagasawa et al. 1994). Newbold et al. (1989) have suggested the role that protozoa play in ruminal peptide metabolism. These authors have observed that ciliates are important in the uptake and cleavage of dipeptides. Thus, it appears that defaunation should markedly inhibit this final

stage in peptide metabolism and lead to an increase flow of intact dipeptides to the small intestines. Nevertheless, they pointed out that in vivo, due to increased bacterial population with defaunation this effect could be suppressed.

Wallace et al. (1987) concluded that the contribution of ciliates to deaminase and peptidase activities is higher than the bacterial contribution; nevertheless, some protease and dipeptidase activities of ciliates in the rumen are similar to bacterial. Therefore, the overall effect of protozoal suppression on N metabolism will depend upon the relative importance of each hydrolytic step on overall ammonia formation. Generally, the amount of feed protein degraded in the rumen decreases when animals are defaunated (Ushida et al. 1984; Ushida and Jouany 1985; Kayouli et al. 1986; Ushida et al. 1986; and Hsu et al. 1991a).

Coleman (1975) proposed that ciliates utilize only about half of the ingested N; the rest is expelled as short-chain peptides and free amino acids. This may explain why the free amino acid concentration in the rumen decreases with defaunation (Itabashi and Kandatsu 1975; and Faciola et al. 2004). It has been estimated that 1% of the cellular N is excreted per hour by entodiniomorphids in the form of amino acids, peptides and ammonia (Abou Akkada and Howard 1962).

Muszynski and Michalowski (1979), testing the effect of different sources of protein in entodinimorphids in vitro, found that these ciliates do not metabolize soluble protein. This work has confirmed a negative correlation between protein solubility and the rate of protein degradation exhibited by isolated protozoa. Moreover, the authors have observed that ruminal ciliates do not grow unless insoluble proteins are supplied.

In vitro studies indicated that the production of ammonia from fish meal, soybean meal, lupins and peanut meal was significantly greater in ruminal contents obtained from faunated sheep than defaunated sheep. In the same studies protozoa had no effect on

ammonia production when casein was used as a substrate (Ushida and Jouany 1985; and Jouany et al. 1992). These results may be explained by the high capacity of entodiniomorphid ciliates, which represented 99% of the population tested, for ingesting and digesting insoluble particles, and by their low activity toward highly soluble proteins such as casein.

Protozoa digest the insoluble engulfed protein inside their cells by use of active proteases present at high concentration; these enzymes are not diluted by being secreted into the rumen fluid. Epidinia, unlike the others ciliates, release proteases into the medium (Coleman and Laurie 1974).

Lockwood et al. (1988) reported multiple forms of proteases in holotrichs. Onodera and Kandatsu (1970) also found that isolated holotrichs rapidly degraded casein; however, no urease activity was found in starved, antibiotic-treated ciliate protozoa (Onodera et al. 1977).

Generally, protein solubility is considered a deciding factor in ruminal protein degradation (Craig and Broderick 1981; and Craig et al. 1987). Ushida et al. (1991) suggested that the role of holotrichs in protein digestion is negligible; they pointed out that bacteria mainly digest the soluble protein. In addition, Nugent and Mangan (1981) suggested that soluble proteins adsorb on the bacterial surface before being attacked by proteolytic enzymes.

Despite the capacity of entodiniomorphid to engulf insoluble proteins and the ability of holotrich to degrade soluble proteins, there may be a synergistiz effect of the different genera in the rumen. Onodera and Yakiyama (1990) suggested that holotrichs may produce some casein coagulating substances that render casein sensitive to entodiniomorphids.

Marcin et al. (1998), in an in vitro study, found an average rate for wheat protein proteolysis of 0.137 mg ml^{-1} h^{-1} in the protozoal culture medium in comparison with a rate for azoalbumin proteolysis of 0.048 mg ml^{-1} h^{-1} in the supernatant during a 24h of incubation. These results suggest the higher proteolytic activity of ciliates on insolubles proteins. In an in situ study Ushida and Jouany (1985) showed that the potential degradable fraction of insoluble protein in soybean meal increased 11% when mixed protozoa were inoculated into defaunated sheep.

It is well accepted that protozoa become progressively more important in protein utilization the more insoluble the protein becomes. In order to verify this hypothesis, Ushida et al. (1986) compared the rates of degradation of feed protein in faunated and ciliate-free sheep given two isonitrogenious diets. The first diet consisted of alfalfa hay in which the protein had low solubility. The feed N digestibility increased by 95% with protozoa inoculation. The second diet consisted of NaOH-treated straw and urea, in which the N had high solubility; the feed N digestibility increased only 32% by protozoa inoculation, threefold lower than when insoluble protein was available.

It appears that physical form is one of the most important factors influencing the relative contribution of protozoa and bacteria to protein degradation. Mixed ruminal bacteria adsorb proteins into the cell wall and hydrolysis occurs at this site (Wallace 1985). In this process, solubility and the primary sequence of amino acids are the most important determinants of proteolysis. On the other hand, engulfment is the main factor determining the extent of protozoal proteolysis. Therefore, availability and size of bacteria may be important issues regarding protozoal predation.

It should be mentioned that at some point, that the increase in the number of bacteria in the rumen of defaunated animals, particularly *Prevotella ruminicola* (Kurihara et al. 1968; and Kurihara et al. 1978) which exhibits intense proteolytic activity, will

partially compensate for the disappearance of protozoa and limits the effect of defaunation.

1.2.2 Ammonia Concentration in the Rumen

The ruminal ammonia concentration derives mainly from protein breakdown by microorganisms and frequently exceeds microbial growth requirements. Excess ammonia is absorbed through the rumen wall and most will eventually be excreted as urea in the urine. This ammonia loss contributes to environmental pollution (Meisinger and Jokela 2000) and decreases efficiency of N utilization by the animal (Tamminga et al. 1979; and Leng and Nolan 1984).

Wallace et al. (1987) and Wallace and McPherson (1987) reported higher deaminase activity in faunated compared to defaunated animals, especially when small entodiniomorphids were present. In vitro, Hino and Russell (1987) reported that the activity of deaminases in protozoal extracts were 2-3 times higher than in bacterial extracts. Concentration of ammonium ions in the environment may regulate the N metabolism of protozoa. An increase in ammonium ions in the rumen favors the direct assimilation of free amino acids and reduced proteolysis (Ahuja and Sarmah 1979). These mechanisms help to explain the drop in ammonia concentration of defaunated animals, even if its incorporation into bacterial cells is not modified by ciliate disappearance (Itabashi and Kandatsu 1975).

Veira et al. (1983) found a lower concentration of plasma urea N in defaunated sheep compared to partial or completely faunated sheep (2.6, 6.6, and 8.2 mg/dl, respectively). This is in agreement with lower ruminal ammonia, often reported in defaunation studies.

In comparing of ciliate-free and faunated animals, one common observation has been a higher concentration of ruminal ammonia in faunated ruminants. Hsu et al.

(1991a) studied the effect of defaunation on utilization of different N supplements varying in ruminal degradability on sheep and found a decrease in ruminal ammonia concentration in all treatments. According to Itabashi and Kandatsu (1975), the decrease in the concentration of NH_3 observed in the rumen of defaunated animals is not caused by a greater utilization of NH_3-N by bacteria, since the half-life of NH_3-^{15}N does not differ between faunated and defaunated animals. However, faunated animals usually have a higher concentration of free amino acids in the rumen, and in this condition, ruminal bacteria may use less ammonia-N. Williams and Coleman (1992) summarized 38 studies where the ruminal ammonia concentration was significantly reduced by defaunation. This effect is usually associated to protozoal ingestion and degradation of bacteria protein (Broderick et al. 1991).

The selective retention reported for protozoa within the rumen may contribute to the ruminal ammonia pool since the death and lysis of the cells release partiality digested proteins, as well as amino acids and peptides.

1.2.3 Microbial Recycling within the Rumen

Microbial recycling within the rumen is the result of both protozoa and bacteria lysis and degradation. The recycling of microbial protein releases amino acids, peptides and ammonia into the medium. Microbial protein synthesized in the rumen contributes up to 80% of the protein entering the small intestine, even though up to 50% of the microbial protein is degraded to NH_3-N within the rumen (Leng and Nolan 1984). As a consequence of bacterial predation and subsequent protozoal lysis, N recycled within the rumen – corresponding to the difference between total incorporation of N into microbial cells (total protein synthesis) and the net outflow of microbial N (net protein synthesis) – is much greater in faunated than in defaunated animals (Jouany 1996).

The absence of ciliates strongly affects the numbers of bacteria in the rumen. Eadie and Hobson (1962) showed that when lambs, which had been kept ciliate-free from birth, were inoculated with rumen ciliates, the number of small bacteria declined almost three-fold. Coleman (1975) reported that a single protozoan could take up 10^2 – 10^4 bacteria/h. Applying a value of 10^9 bacteria/ml to the rumen, these estimates indicate that predation could almost renew the entire bacteria biomass every hour in a rumen harboring a protozoal concentration of 10^5-10^6 ml. Nevertheless, it must be pointed out that these results were obtained in vitro; however, in vivo protozoa also engulf feed particles which reduces their capacity to engulf bacteria. Moreover, because the majority of bacteria colonize feed particles rather than existing as freely floating, and these bacteria are probably not as accessible for engulfment.

The rate of bacterial engulfment is highly dependent on bacterial population density, increases from 340 to 4070 bacteria/protozoan/h, on an increasing bacterial population density from 10^9 to 10^{10}/ml was reported by Williams and Coleman (1992). Williams and Coleman (1992) suggested that two parameters probably determine the rate of uptake of various bacterial species by protozoa. The first is the number of bacteria taken up per hour from an infinitely dense bacterial suspension. This parameter probably measures the maximum ability of a protozoan to engulf bacteria and may depend on the size and shape of the bacterium. The second parameter is the volume of medium cleared of bacteria from an infinitely dilute bacterial suspension. This parameter probably measures a protozoan's ability to seek, find, and catch bacteria, and may depend on the attraction between the bacterium and the protozoan. The authors pointed out that the first parameter more likely applies to the rumen conditions.

The effects of ciliates on rumen metabolism vary among species. Regarding protein degradation, small entodiniomorphids have been shown to be by far the major

contributor to bacterial protein turnover in the rumen (Wallace and McPherson 1987). The composition of ruminal bacteria also is altered by the presence of protozoa. Itabashi and Katada (1976a), and Ushida et al. (1987) observed that the numbers of small gram-negative rods, selenomonas-like bacteria and gram-negative cocci were higher in defaunated animals, whereas gram-positive strains were unchanged. Kurihara et al. (1978) reported a decrease of amylolytic bacteria after defaunation. Ushida et al. (1991) reported that total viable counts of amylolytic, pectolytic, and cellulolytic bacteria increased after defaunation. Thus, changes in digestion and fermentation patterns resulting from defaunation could be attributed to modifications in the distribution of rumen bacterial species as well as to the direct effect of the absence of ciliates.

Ushida et al. (1991) suggested that the relationship between protozoa and other microorganisms depends on the nature of the diet and types of organisms. Moreover, Williams and Coleman (1997) suggested that the physical form of bacteria, the nutritional status and size of protozoa, density of populations, and the environmental conditions in the rumen also affect the predator-prey relationship. Coleman and Sandford (1979) concluded that in tropical conditions, the optimum pH for bacteria uptake by protozoa is 6-7. In the same study, they also have shown that salt concentration has an effect on protozoal predation. Other particulate matters such as starch have an effect on the rate of bacteria engulfment by the ciliates. Williams and Coleman (1992) reported a decrease in bacterial uptake by protozoa when an excess of starch is added, however, uptake was completely abolished with no added starch.

Williams and Coleman (1992) summarized the uptake and digestion of individual bacteria by different protozoa species. They concluded that *Butyrivibrio fibrisolvens* and *Proteus mirabilis* tend to be engulfed preferentially and *Bacillus megaterium*, *Butyrivibrio fibrisolvens*, and *Selenomonas ruminantium* were digested most rapidly.

These results mean that after the inoculation of a protozoa-free rumen with a mixed protozoal population, the population densities of *Butyrivibrio fibrisolvens* and *Selenomonas ruminantium* will tend to selectively decrease. Whether this will affect fiber digestion and fermentation in the rumen is not clear.

Protozoa also interact within species and with fungal population. There is evidence that cannibalism and predation occurs between different species of protozoa (Williams and Coleman 1992). The complexity of ecological relationships between rumen microbes becomes difficult to accurately predict the effects of the withdraw of one or more species from the media. Williams and Coleman (1997) elaborated on predatory and metabolic interactions between protozoa and fungi in the rumen. Defaunation can result in an increased zoospore density (Orpin 1977) although, in other studies, defaunation has had no significant effect on zoospore numbers (Williams and Withers 1991). Scanning electron microscopy clearly showed that some protozoa are able to ingest fungal rhizoids and occasionally sporangia (Williams and Coleman 1997). Subsequent fungal digestion has been confirmed by extensive ^{14}C solubilization when ^{14}C-labelled *Piromonas communis* was incubated with individual species of ruminal species of ciliates (Williams and Coleman 1997). Glucosamine and amino acid release from *P. communis* by mixed ruminal ciliates was also seen as an indicator of protozoal chitinolytic activity and turnover of fungal protein (Morgavi et al. 1994a; and Morgavi et al. 1994b). Fungal protein turnover is reduced by defaunation (Newbold and Hillman 1990).

Koenig et al. (2000) indirectly studied the effect of protozoa on microbial N recycling by determining intraruminal recycling of NH_3-N from kinetic analysis of ^{15}N enrichment in the ruminal NH_3-N and plasma urea N pools following a pulse dose of the tracer into each of these pools. With this technique, any release of amino acids and

peptides through the lysis and degradation of bacteria and protozoa that does not resulting N passage through the ruminal NH_3-N pool underestimates the extent of microbial N recycling. Although they found an increase in ruminal bacteria pool, microbial N flows to the duodenum, and microbial efficiency after defaunation, they did not observed difference on intraruminal recycling of microbial N between faunated, defaunated, and refaunated sheep. Furthermore, they reported considerable variation among animals. In contrast to these results, Firkins et al. (1992) reported intra-ruminal recycling of microbial N ranging from 75 to 90% in dairy cows. In addition, in the model of Aharoni et al. (1991), microbial N recycling in faunated animals is set to 22 to 62%, depending on the protein source of the diet.

There is little information available concerning protozoa generation time; this may become an important issue because these microbes have a lower passage from the rumen than expected based on their ruminal mass and because their lysis can significantly contribute to the overall microbial recycling in the rumen. Warner (1962) estimated the generation time of mixed entodiniomorphids at 5.5 to 58h in sheep. Potter and Dehority (1973) calculated the generation time at 13.5 to 37.3h in sheep. Williams and Withers (1993) estimated the generation time at 9-10h for entodiniomorphids during the initial stages of refaunation in sheep. Coleman (1979) obtained, in vitro, values ranging from 6 to 38h for the generation time of different species of entodiniomorphids. Dehority (1998) reported a mean of 25.2h for the generation time of *Epidinium caudatum* and *Entodinium caudatum*. In the same work, the author pointed out that these species are capable of doubling in approximately 12-13h, allowing these organisms to maintain themselves in the rumen under conditions of rapid ruminal turnover.

It is not clear whether fluid or particulate phases better represent protozoal flow. Hungate et al. (1971) estimated that 80% of ruminal protozoa pass with the fluid phase,

which has a turnover time of about 6.25h, equivalent to a mean passage rate of 0.160/h (Hristov and Broderick 1996). Hungate (1966) concluded that, for any organisms to survive in a continuous system, its generation time must be equal or less than .69 times the turnover time. Thus, the generation time would need to be 4.31h for these protozoa to maintain themselves in the rumen.

1.2.4 Microbial Synthesis in the Rumen

Despite the ability of protozoa to digest exogenous feed proteins, Williams and Coleman (1997) pointed out that the main role of the ciliates is to digest bacterial protein. Protozoa have high lysozyme activity, which effectively breaks down the bacterial cell wall. They also have chitinase, which presumably enables them to utilize fungal biomass in a similar way (Morgavi et al. 1994a; and Morgavi et al. 1994b).

Increases in both bacterial growth and efficiency of microbial protein synthesis have been observed with defaunation in many in vivo experiments (summarized in Ushida et al. (1991)). The reason for increasing microbial protein synthesis efficiency is due to a higher ruminal outflow of microbial protein and to a lower organic matter digestion, since the microbial efficiency is usually expressed as grams of N incorporated into microbial protein per kilogram of organic matter apparently digested in the rumen.

Most research attributes the increase in efficiency of microbial synthesis to the reduction of bacterial recycling within the rumen after defaunation (Demeyer 1981). However, Ushida et al. (1991) pointed that there is disagreement as to why the efficiency of bacterial protein synthesis is lower in the presence of protozoa. It has been argued that the reduction in bacterial population density after faunation could be associated with a higher metabolic activity of the remaining bacteria and thus this might explain the common enhancement of VFA production in the faunated state.

The effect of protozoa on the efficiency of bacterial growth represented by NH_3-N utilization is not conclusive. Itabashi and Kandatsu (1975) reported a higher ^{15}N uptake during the first 10 hours after introducing ^{15}N-ammonium citrate and a more rapid decrease in ^{15}N-concentration in bacteria after this period in the rumen of faunated versus defaunated goats. Other studies did not find differences on bacterial NH_3-N enrichment after defaunation (Koenig et al. 2000; and Hristov et al. 2004).

Some of these conflicting results might be related to different definitions of microbial growth. Van Nevel and Demeyer (1977) introduced two definitions of microbial synthesis, net and total. The first corresponds to the microbial protein that is flowing from the rumen, whereas the second corresponds to the microbial protein synthesized in the rumen, including microbial protein recycled within the rumen. Applying these concepts, Demeyer and Van Nevel (1979) reported an increase in net synthesis by defaunation but total synthesis remained unchanged or was only slightly increased. Measuring the efficiency of microbial synthesis in terms of grams of microbial true protein synthesis per gram of N intake may be a more precise way of expressing the true N utilization by the rumen microbes.

Koenig et al. (2000) concluded that defaunation improves the intraruminal metabolism of N by increasing ruminal bacterial biomass synthesis and flow of bacteria to the intestine. They reported an increase of 89% on microbial efficiency for defaunated sheep. Ushida et al. (1986) estimated bacterial N flow using diaminopimelic acid (DAPA), nucleic-acid purine bases (PB), and ^{35}S incorporation. They observed higher bacterial N flow and higher efficiency of bacterial protein synthesis in defaunated animals. In this study, the three different markers showed good agreement, particularly in these defaunated animals.

Based on the knowledge that defaunation increases bacterial density population up to fourfold and reduces ruminal ammonia concentration up to 60%, Hsu et al. (1991a) hypothesized that the bacterial requirements for NH_3-N might not have been met in some defaunation studies, especially for fiber digestering microbes, and this may account for lower fiber digestion commonly observed with defaunation. Furthermore, very low ammonia levels may be limiting to microbial growth in the rumen. Some studies have been reported that the quality of protozoal protein is better than that of bacterial protein (McNaught et al. 1954; Purser and Buechler 1966; Bergen et al. 1968; Ibrahim and Ingalls 1972; Williams and Dinusson 1972; and Williams and Dinusson 1973). Onodera and Kandatsu (1973) demonstrated the synthesis of lysine from 1,7-diaminopimelate by ciliates. The synthesis of other amino acids, such as tryptophan and phenylalanine, by ruminal ciliates also has been reported (Onodera 1990; and Onodera et al. 1997).

1.2.5 Nitrogen Flow from the Rumen

Measurement of the contribution of protozoal N to total microbial N leaving the rumen has presented major problems because of the lack of a suitable reference marker. An amino acid peculiar to protozoa, 2-amino-ethyl phosphoric acid, has been suggested for use in such studies (Horiguchi and Kandatsu 1959; and Abou Akkada et al. 1968). John and Ulyatt (1984) studied the feasibility of phosphatidyl choline as a specific marker for protozoa. Most of the markers proposed have shown several problems such as, contamination from feed and others microbes, non-representative sampling, high variability, uneven distribution or assimilation, and difficulty in the determination.

It was assumed in the past that protozoa made a significant contribution to the protein nutrition of their host. However, the issue that ciliates are selectively retained in the rumen (Weller and Pilgrim 1974; Jouany and Senaud 1978; Harrison et al. 1979; Coleman et al. 1980; Leng et al. 1981; John and Ulyatt 1984; Punia and Leibholz 1984;

and Michalowski et al. 1986) and thus do not contribute to the protein supply of the host in quantities proportional to their ruminal mass, has led to re-evaluation of their contribution to ruminal protein nutrition.

According to Jouany et al. (1988), protozoa provide only about 20% of the total microbial N entering the duodenum of the ruminant, which does not reach the level expected given the protozoa/bacteria biomass ratio in the rumen. Protozoa can comprise half of the microbial biomass (Michalowski 1979; and Jouany et al. 1988). The difference observed between protozoa N concentration in the rumen and duodenum is attributed to the selective retention of ciliates in the rumen. The rate of outflow of protozoa from the rumen is inversely linked to the retention time of solids particles (Crawford et al. 1980; and Meyer and Mackie 1986), but is not influenced by the dilution rate of the fluid phase in the rumen (Michalowski et al. 1986) or by the numbers of protozoa (Meyer and Mackie 1986). The matrix of solid particles in the rumen probably has a major role in the sequestration of protozoa (Godeau et al. 1985), which are attracted by release of fermentable carbohydrates (Orpin and Letcher 1978; and Murphy et al. 1985) after cell-wall rupture. Moreover, certain ciliates such as Isotricha spp. and Epidinium spp. can attach to food particles (Bauchop and Clarke 1976; Orpin and Letcher 1978; and Grain and Senaud 1984) and others, like Isotricha spp., attach to the rumen mucosa (Abe et al. 1981). Steinhour et al. (1982) and Punia et al. (1992) reported that protozoal protein was 22 to 41% of ruminal protein but only 11 to 20% of the total microbial protein flow.

Veira et al. (1983) found a sharp decrease in amino acid flow to the small intestine of sheep after inoculation with ciliates. According to the authors, the negative effect of protozoa on AA flow was likely related to their inefficient utilization of N and preferential retention in the rumen, as well as to changes in digestion in the rumen. Ivan et al. (2000a), evaluating the inoculation of fauna-free wethers with different ciliate genera,

found that compared to fauna-free animals, the flow of bacterial N in wethers containing Entodinium and total fauna decreased 25 and 23%, respectively, on a hay crop diet but the reduction was 46% when fed corn silage. Feed differences may influence these results by altering protozoa population and density. In the same trial (Ivan et al. 2000a) inoculation with total fauna and Entodinium decreased duodenum flow of non-ammonia N (NAN) by 16% and 17% and total amino acids (AA) by 20% and 19%, respectively. The authors affirmed that Entodinium sp. might have the most negative effect of all the main ruminal ciliates on duodenal flow of AA. In another experiment, Ivan et al. (2000b) reported a decrease in the flow of bacterial N of nearly 45% after inoculation with ciliates. The reduction in NAN flow was higher than 20% and the reduction in the total AA flow reached 30% of the various genera, Holotrich protozoa showed the smallest impact on N metabolism.

Shabi et al. (2000) determined the flow of amino acids from protozoal origins using linear programming. They found that AA flows to the abomasum and the proportion of protozoal AA in abomasal flow were increased by 100.0 and 72.9%, respectively when cows were fed diets with low rumen degradable protein compared with high rumen degradable protein diets. Protozoal CP was decreased when high rumen degradable protein diets were fed and provided from 6.9 to 18.3% of the crude protein flowing to the abomasum. These data suggest that protozoal growth is more active when insoluble protein is available.

Jouany (1996) summarized 26 studies, most of which reported an increase on duodenal N flow with defaunation. Generally, defaunation has a positive effect on the duodenal N flow, by increasing the flow of both feed N undergraded in the rumen and microbial N synthesized in the rumen, in contrast to other rumen manipulations using feed additives (e.g. ionophores) which act positively only on one parameter.

Itabashi and Katada (1976b) reported a higher concentration of free amino acids in the plasma of defaunated animals. Two hypotheses may explain this response: an increase in the supply of α-amino N to the duodenum of defaunated animals, as discussed above, or a lack of the supply of one or more essential amino acids that may limit the utilization of others by the defaunated animals. Protozoal protein has a higher intestinal digestibility than bacteria (McNaught et al. 1954) and the protozoal cell wall is not resistant to the action of enzymes, which allows better utilization of intracellular proteins (Jouany et al. 1988). Moreover, the levels of important amino acids such as lysine and phenylalanine are higher in protozoal than bacterial protein (Purser and Buechler 1966; Bergen et al. 1968; Ibrahim and Ingalls 1972; Williams and Dinusson 1972; and Williams and Dinusson 1973). Indeed, while defaunation could increase the AA flow, some have speculated that the complete absence of protozoa could lead to a specific deficiency of certain amino acids; however, this may not happen since the limiting AAs are likely to increase with the others.

1.2.6 Nitrogen Excretion

N excretion is a major world concern these days. More than a waste of energy for animals and a high cost nutrient, excess of N excretion contributes to environmental damage, such as the greenhouse effect, denitrification, leaching and runoff (Misselbrook et al. 1995). It has been estimated that 90% of US ammonia emissions come from agriculture and 90% of those emissions are due to manure from livestock enterprises (Meisinger and Jokela 2000). Jouany (1996), summarized seven studies that showed a lower N excretion in urine after defaunation; this was associated mainly with the lower ruminal ammonia concentration and possibility with increased capture of blood urea for microbial synthesis. On the other hand, fecal N excretion increased after defaunation. The most acceptable explanation is that the lower ruminal digestion of structural

carbohydrates usually observed after defaunation is compensated for by greater digestion in the large intestine (Ushida et al. 1991). As a consequence, there is more microbial protein formed in the large intestine and greater out flow of fecal microbial N. Thus, differences in total N losses between faunated and defaunated animals largely disappear and the net effect of defaunation is the shift of N excretion from the urine to the feces. However, changing the pattern of N excretion from urine to feces, even with the same amount of N excretion, might be useful since environmental problems regarding feces N concentration are less damaging than N emission from urine, which is the most environmentally labile form of excreted N (Varel et al. 1999).

1.3 Effects of Protozoa on Carbohydrates Metabolism

1.3.1 Effect of Protozoa on Digestion of Non Structural Carbohydrates

Rumen defaunation has no effect on the overall digestibility of starch and sugars in the digestive tract of the ruminant (Jouany et al. 1978). However, a rapid bacterial fermentation, initiated by an abundant energy supply, will result in considerable lactic acid production, a condition which may impair rumen function (acidosis) and microbial N yield in defaunated animals receiving diets rich in starch and soluble sugars. It is widely believed that efficient uptake of starch and soluble sugars by protozoa prevents their rapid fermentation by bacteria, thereby limiting the decrease in pH and lactic acid production, maintaining a more stable ruminal environment.

Holotrich protozoa are attracted to sugars by chemotaxis (Orpin and Letcher 1978). They assimilate sugars non-selectively and store them as amylopectin, which is metabolized slowly to produce volatile fatty acids and gasses long after the sugars were initially present in the rumen (Jouany et al. 1978). Amylopectin storage is particularly important because it accounts for 80% of the glucose assimilated by holotrich ciliates

(Prins and Van Hoven 1977; and Van Hoven and Prins 1977). Amylopectin is formed from most sugars except hexosamines and mannose, which are toxic to holotrich protozoa (Sugden and Oxford 1952). Because they have no mechanism regulating their sugar intake, protozoa can burst when excess sugars are available. This is not related to excessive polysaccharide storage, since that mechanism of synthesis is controlled. It results from the accumulation within the cell of acid fermentation products (Williams 1986) formed from ingested sugars in excess of those incorporated for polysaccharide storage.

As far as is known, all the entodiniomorphid protozoa engulf starch grains, although the smaller protozoa only take up smaller particles, such as those found in rice. The grains are taken up through the esophagus or cytostomal invagination into vesicles in the endoplasm (Williams and Coleman 1992).

Of the plant polysaccharides, starch is the preferred substrate for protozoa and the ability to ingest starch granules is highly dependent on the size of the granule and the protozoan cell (Ushida et al. 1991).

The absorption rates of glucose is higher by Isotricha spp. than by entodiniomorphid protozoa (Williams and Coleman 1997). When cattle are fed molasses and sugarcane, holotrich protozoa proliferate and become the major species (Valdez et al. 1977; and Boodoo et al. 1978).

Several carbohydrate metabolizing enzymes were found in ruminal protozoa (Williams and Coleman 1992). It has been shown that the specific amylolytic activity in the rumen is higher in the protozoal fraction (Williams and Strachan 1984; and Coleman 1986b). Defaunation can induce a loss of the amylolytic activity in the rumen (Ushida et al. 1991) and this could lead to a lower fermentation capacity, decreasing VFA production. However, increases in amylolytic bacteria after defaunation can partially or

even fully compensate for the disappearance of protozoal amylolytic activity. This may be the explanation for the absence of effect on apparent starch ruminal digestibility in vivo after defaunation.

The amount of carbohydrates stored by ciliates during the 4h following feed intake could represent between 30-40% of their dry matter (Jouany and Thivend 1972). Based on this estimate, the passage of even a small quantity of protozoa would contribute to a significant amount of amylopectin to the small intestine.

1.3.2 Effect of Protozoa on Digestion of Structural Carbohydrates

Many entodiniomorphid protozoa are able to growth in vitro on a dried-grass powder substrate (Coleman et al. 1980), suggesting that they are able to use fiber for their own nutritional needs. The presence of hemicellulolytic activity was demonstrated in early studies (Bailey et al. 1962; Abou Akkada 1965; Bailey and Gaillard 1965; and Naga and el-Shazly 1968). Ushida et al. (1991) reported that protozoa have a more obvious role in hemicellulose digestion than in cellulose digestion. Furthermore, except for *Dasytricha ruminantium* and small entodinia, all protozoal genera produce enzymes active against plant polysaccharides (Williams et al. 1984; Williams and Strachan 1984; and Williams and Coleman 1985). As a consequence, the positive effect of natural fauna on hemicellulose digestion is virtually independent of fauna type.

Rumen lignocellulose digestion is less clearly affected by protozoa than hemicellulose digestion (Ushida et al. 1991). These authors report that ruminal lignocellulose digestion is enhanced by protozoa when the diet is supplemented with starch, suggesting that there are interrelationships between dietary starch, presence of protozoa, and cellulose digestion. In the absence of ruminal protozoa, amylolytic bacteria, which grow faster than cellulolytic bacteria, overcome the latter and produce an inadequate environment for fiber digestion especially due to lactic acid production. As

evidenced by bacteriological surveys, the concentration of amylolytic bacteria is reduced when protozoa are present (Ushida et al. 1991). Moreover, entodinimorphid protozoa create favorable conditions for fiber digestion by metabolizing lactic acid (Newbold et al. 1986).

Coleman (1986a) compared the specific activity and distribution of carboxymethylcellulase (CMCase) in the sheep rumen harboring defined fauna. CMCase activity in the protozoal cytoplasmic fraction represented 5 to 90% of total activity in the rumen. Inoculation with Entodinium caudatum increased total CMCase activity. This increase was probably due to an increase in the number and activity of cellulolytic bacteria because the bacterial CMCase was 2.5-fold more active in inoculated than in defaunated rumen fluid. Again, this suggests the synergism between noncellulolytic small entodinia and cellulolytic bacteria. Hristov et al. (2004) found a decrease in CMCase after reducing the protozoal population by 91%. On the other hand, large entodiniomorphids seem to show an antagonism toward cellulolytic bacteria. Ushida et al. (1991) stated that the number of cellulolytic bacteria is decreased by large entodimorphid protozoa. Hence, the inoculation of protozoa, in general, increases the total CMCase activity of rumen fluid, but the effect of large entodiniomorphid protozoa should be distinguished from that of small noncellulolytic entodinia.

Unlike entodiniomorphid protozoa, holotrich protozoa have a detrimental effect on fiber digestion, mainly attributed to the high lactic acid production by those genera. Jouany et al. (1988) observed low pH and high lactic acid concentration in sheep when Isotrich ssp. were the only species inoculated. However, when inoculated with Entodinium ssp. or *P. multivesiculatum*, the number of Isotricha ssp. fell, lactic acid concentration decreased and pH increased. Thus, entodiniomorphid protozoa can have a positive direct (large cellulolytic species) or indirect (small noncellulolytic entodinia)

effect on rumen lignocellulose digestion, whereas holotrich protozoa can be considered parasites in terms of fiber digestion.

Romulo et al. (1986) detected high cellulolytic activity in the rumen of defaunated sheep fed a low-quality straw-based diet. In those experiments, defaunation increased viable zoospore counts of rumen fungi to a very high level. Such a large fungal population might be responsible for an enhanced rumen cellulolysis. Fungal cellulolytic activity in defaunated sheep rumen may have been as great as 70% of bacterial activity when viable zoospore numbers were in order of 10^3/ml (Ushida et al. 1989). Defaunation probably increases the fungal population size, and fungi can partly compensate for a decrease in cellulolytic activity induced by defaunation (Williams and Coleman 1992).

Most in vivo studies have shown that defaunation lowers cell-wall carbohydrate digestion in the total digestive tract by as much as 5-15%. This drop, however, is highest (28%) when digestion is measured at the duodenum (Jouany et al. 1988). This indicates that there is a shift in cell-wall carbohydrates digestion from the rumen to the hindgut in defaunated animals. This shift probably accounts for the increased fecal N and fecal energy (Ushida et al. 1991; and Jouany 1996).

Although the nature of the diet does influence the number of rumen ciliates (Jouany et al. 1977), the effects of defaunation on cell-wall carbohydrates cannot be readily attributed to diet or to the size of protozoal population (Jouany et al. 1988). Hungate (1966) has shown cellulase activity in ruminal protozoa. Williams and Coleman (1985) found hemicellulase activity in different species of ciliates. Moreover, Orpin and Hall (1983); and Orpin (1984) have found that ciliates have enzymes that can degrade pectic substances. Although the enzymes of ciliates can degrade cell-wall carbohydrates, the direct effect of protozoa on cell-wall digestion is probably low (Jouany et al. 1988).

The same authors pointed out that the major effect is indirect and can be attributed to the effect of protozoa on cellulolytic bacteria.

Orpin (1984) suggested that release of cell contents after cell lysis plays a role like chemoattractants for some ciliates, and by this mechanism, they are able to locate freshly ingested materials. Moreover, different strategies of attachment by ruminal protozoa have been reported (Orpin and Letcher 1978; Bauchop and Clarke 1976; and Bauchop 1980).

Therefore, presence of protozoa seems to be important to ruminal fiber digestion as a whole. Due to their ability to digest structural carbohydrates by themselves, by affecting fiber digesting bacteria and fungi, or by creating or maintaining an adequate ruminal environment for fiber digestion.

1.4 Effects of Protozoa on Ruminal Fermentation

1.4.1 Effects of Protozoa on Ruminal Volatile Fatty Acids

Most literature has reported a decrease in total VFA production as a consequence of defaunation. This has been usually attributed to the reduction in carbohydrate fermentation, especially cell-wall carbohydrates, which was discussed earlier. Moreover, the role protozoa play in maintaining pH stability by slowly fermenting sugars and starch and metabolizing lactic acid may have an impact on total VFA production. The effect of defaunation on the proportions of VFA is variable, although the results of experiments frequently have shown an increase in molar proportion of propionate at the expense of butyrate and acetate. Acetate and butyrate are produced by ruminal ciliates during carbohydrate fermentation (Williams and Coleman 1997). Therefore, this may explain the reduction in the molar proportion of these acids after defaunation.

Propionate has an inhibitory effect on ciliates (Kobayashi and Itabashi 1986). This could partly explain the depression of protozoal population observed when animals are given a very high starch diets.

Jouany et al. (1988) suggested that defaunation leads to the formation of butyrate via acetyl-CoA, as well as to a stimulation of the conversion of acetate into butyrate. According to the same authors, this pathway could explain the shift in VFA towards production of butyrate, which is sometimes observed in defaunated animals given diets rich in carbohydrates, even though the majority of the literature has reported a decrease in butyrate production. Veira (1986) suggested that because of the inconsistency of the results of defaunation on VFA proportions, we should not assume that ciliates per se are responsible for the observed differences. This author suggested that changes in the bacterial population, probably will play a role in the pattern of VFA production.

There is little information on the effects of defaunation on branched chain VFA (BCVFA). However, due to the reduction in proteolysis and amino acid concentration in the rumen commonly reported after defaunation, it is expected that the absence of protozoa would reduce ruminal BCVFA. This may be especially important to cellulolytic bacteria which appear to require these acids (Hungate 1966).

Some of the experiments that have shown an increase in total VFA production attributed this to the increase in bacterial population in the rumen, which is commonly observed in defaunation studies.

1.4.2 Effects of Protozoa on Ruminal pH

It has been shown that entodiniomorphid protozoa actively metabolize lactic acid in the rumen (Newbold et al. 1986). In contrast, holotrich protozoa produce lactic acid (Williams and Harfoot 1976), suggesting that the overall effect of protozoa on ruminal pH might be different depending on the main type of ciliate present in the rumen. Moreover,

Hristov et al. (2001), found that reducing ruminal protozoa population by 42% did not alter ruminal L-lactate. The most accepted effect of protozoa on ruminal pH is their ability to slow the rate of fermentation of sugars and rapid non-structural carbohydrates, and reduce the availability of those carbohydrates to bacterial fermentation, thereby helping to maintain a more constant pH.

1.5 Most Common Methods of Defaunation

Jouany et al. (1988) divided the experimental methods for artificial defaunation of the rumen in three major categories: Isolation from birth, chemical treatments, and emptying and treatment of rumen contents.

The first method consists in separating the young animal from its dam soon after birth and preventing it from having contact with other faunated ruminants. According to Ivan et al. (2000a), it was possible to maintain a sheep flock over 20 years in this fauna-free state. Therefore, this method removes from the experiment any carry-over effects from the defaunation technique. Moreover, it causes less harm to the animal compared to the two other methods. On the other hand, this method is applicable only under experimental circumstances.

In the second method summarized by Jouany et al. (1988), chemicals are introduced into the animal's rumen, either via an esophageal probe or through rumen cannulae, in order to destroy the ciliate population. Use of several different chemicals were reported, including cooper sulfate, dioctyl sodium sulfosuccinate, alcohol ethoxylate, alkanates, and calcium peroxide. The problems involved in the administration of these chemicals are their unreliability and toxicity, which can even lead to animals death.

Finally, the third method summarized by Jouany et al. (1988) consists in emptying the rumen and treating the contents to kill the protozoa before contents are replaced.

Some of the procedures used to treat the rumen content include heating, freezing, washing the rumen walls with chemicals such as formaldehydes (Hungate 1966). According to the author's summary, this method is reliable, although it requires surgically cannulated animals and can only be applied under experimental conditions.

Among the three major approaches proposed by Jouany et al. (1988), only the second can be applied under practical feeding conditions, even though some of these additives are toxic or have other limitations, such as being unpalatable and difficult to deliver. Furthermore, researchers have also considered other approaches such as fasting (Hungate 1966), feeding high concentrate diets (Franzolin and Dehority 1996), feeding secondary plant compounds (Hristov et al. 1999), or feeding lipids (Newbold and Chamberlain 1988; Matsumoto et al. 1991; Oldick and Firkins 2000; Onetti et al. 2001; and Hristov et al. 2004). The last two approaches should be contemplated as practical and reliable defaunation methods for use under normal feeding managements.

1.6 Lipids as Defaunation Agents

Feeding fats to ruminants is commonly associated with decreased protozoal population in the rumen (Hristov et al. 2004). However, Doreau and Ferlay (1995) have shown that the lipid source and fatty acid composition and saturation may influence the degree of response of the ciliate population. Most of the literature published on ruminal effects of dietary lipids indicates that long-chain, unsaturated fatty acids have a strong antiprotozoal effect (Newbold and Chamberlain 1988; and Doreau and Ferlay 1995). However, Oldick and Firkins (2000) found only a trend for decreasing protozoal counts when increasing the degree of unsaturation of dietary lipids.

Lipids could represent a suitable source of a dietary ingredient used for controlling ciliate population in the rumen because they can be fed routinely. Moreover, most are not harmful to the animals and may even be used to produce food for the organic market.

Newbold and Chamberlain (1988) investigated the effect on protozoal populations in vitro of three sources of fatty acids, Lauric (C12:0), Oleic (C18:0), and Linolenic acids (C18:2). At 100 mg/g, the protozoal activity was 71%, 55%, and 14%, respectively, of the control. In a second in vitro experiment, they tested two oil sources, coconut oil and linseed oil added at 20µl/ml of the medium. The protozoal activity was 74% and 39% of control, respectively. This confirmed the previous results that C18 unsaturated acids have higher toxicity on ciliates.

Newbold and Chamberlain (1988), however, in an in vivo trial using the four rumen cannulated sheep given, via rumen cannulae, coconut oil or linseed oil at: 0, 50, 100 and 150 ml/d reported protozoal counts (x 10^5) of 5.1, 2.5, 0, 0 for the coconut oil and 5.7, 4.2, 1.9, 0.7 for linseed oil. In a second in vivo trial, changing the diet from molasses beet pulp and hay to a barley and hay, the same authors reported counts of 2.1, 0, 0, 0 for coconut oil and 2.56, 1.38, 0.22, 0 for linseed oil. There was no explanation for the differences between in vitro and in vivo results. The authors stated that coconut oil in amounts higher than 50 ml/d reduced intake markedly.

The effect of medium-chain saturated fatty acids on ruminal fermentation and animal performance has been relatively less studied. Protozoa were reported to be completely eradicated from the rumen of goats after two days of feeding lauric acid (5% of dietary DMI) or hydrated coconut oil (6% of dietary DMI) treatment (Matsumoto et al. 1991). Sutton et al. (1983); Newbold and Chamberlain (1988); Matsumoto et al. (1991); Dohme et al. (1999); Machmuller and Kreuzer (1999); Dohme et al. (2000); Machmuller et al. (2000); Sutter et al. (2000); and Machmuller et al. (2003a) clearly established the antiprotozoal properties of coconut oil, which contains approximately 45% lauric acid by CRC (1988).

It is commonly recognized that lipid addition decreases the protozoa numbers. However, effects are variable or not always observed. Doreau and Ferlay (1995) summarized twenty reports showing protozoal counts vared from zero to 136% of control diets with lipid addition.

It is difficult to establish a relationship between degree of saturation, chain length and anti-protozoal effect. Oldick and Firkins (2000) observed a linear decrease in ruminal protozoa concentration as the iodine value of fats increased. According to these authors, ruminal microbes were able to partially adapt themselves to unsaturated fatty acids when the fats were introduced into the rumen in smaller and more frequent meals. They reported that this adaptation often equalized the effects of saturated and unsaturated fatty acids in the rumen and reduced the effects on protozoal counts. In a study conducted by Onetti et al. (2001), there was no effect of fat source (tallow or choice white grease) on ruminal ciliates. Both fat supplements decreased protozoal population; however, the unsaturated:saturated ratio did not influence the results.

Mohammed et al. (2004) fed Holstein steers with α-cyclodextrin-horseradish oil mixture in the concentrate at 2% of DMI. Although the authors reported a reduction in ruminal ammonia concentration, methane formation, and a shift of the molar proportion of acetate to propionate typically observed when ciliates are removed from the rumen, they did not observe any difference on protozoa count. Therefore, under certain conditions, oils seem to have little or no effect on ruminal protozoa.

The effect of oils on protozoal population may be related to the diet. Ueda et al. (2003) found a sharp decrease on ciliate concentration only when linseed oil was added to a concentrate-based diet. No effect was observed on a forage-based diet. Ohajuruka et al. (1991) hypothesized that the negative effect of unsaturated fatty acids on ruminal digestion would be minimized if the diet contained a high proportion of forage, due to the

ability of forage to promote normal rumen function for maximum biohydrogenation. Therefore, the level of lipid added to a forage-based diet may have has to be higher than to a concentrate-based diet, in order to promote the same level of protozoal inhibition.

Lipid supplementation has also been used as an approach to reduce methane formation, since methane represents not only a severe loss of feed energy for the animal (Blaxter and Clapperton 1965) but also a potential contributor to global warming (Moss et al. 2000). Numerous studies have shown that lipid supplementation has an important role in methane production suppression (Van Nevel and Demeyer 1995; Dong et al. 1997; Dohme et al. 1999; Machmuller and Kreuzer 1999; Dohme et al. 2001; Machmuller et al. 2001; and Machmuller et al. 2002). The ciliate population is involved in methane formation in the rumen, mainly due to its association with archaea bacteria (Newbold et al. 1995). Blaxter and Czerkawski (1966) demonstrated in vivo that lauric acid (C12:0) was more potent in suppressing total tract methane production (68% decline) than myristic acid (C14:0), which suppressed methane by 49%. Adding lauric acid to ruminants diets in vitro reduced total methanogenesis (Dohme et al. 2001). Supplementing ruminant diets with coconut oil containing high amounts of C12 and C14 decreased methane production in vitro by up to 88% (Dong et al. 1997) and in vivo by up to 73% (Machmuller and Kreuzer 1999). In the work of Machmuller and Kreuzer (1999), the protozoal population was reduced by 97%. Soliva et al. (2003) suggested that mixtures of lauric and myristic acid could be more effective on methanogenesis suppression than using either alone.

Although many studies have been undertaken, the mechanism of action of lipids against protozoal cells has not been clearly established. The antiprotozoal activity of phospholipid analogues, originally developed as anti-cancer drugs, has been studied on parasites such as Leishmania spp. and Trypanosoma cruzi. (Croft et al. 2003). Therefore,

analogy may be used to try to understand some of the mechanisms involved on protozoal suppression by lipids. Perturbation of ether-lipid metabolism (Lux et al. 1996), inhibition of an enzyme (alkyl-specific acyl-Co-A) involved in lipid-remodeling (Lux et al. 2000), and inhibition of phosphatidylcholine (PC) biosynthesis by preventing the incorporation of methionine into PC, therefore blocking de novo synthesis (Lira et al. 2001), have been suggested as possible reasons for lipid toxicity to protozoa.

The first site of interaction of phospholipid analogues with protozoa is the cell membrane (Croft et al. 2003). Ultra-structural analysis of effects of some phospholipid analogues against protozoal cells showed extensive membrane blebbing, that may lead to membrane dysfunction and ultimately cell death (Santa-Rita et al. 2000). Differences in lipid composition in the cellular membrane may be crucial to the sensitivity of the cell to lipid. Interferences with carrier proteins could also cause depletion of essential nutrients and thus lead to cell death, or at least contribute to growth retardation. Furthermore, Na^+/ K^+-ATPase, another important enzyme in signal transduction, is inhibited by phospholipid analogues (Berkovic et al. 1992). A similar effect of K^+ depletion was observed in ruminal bacteria when exposed to ionophores (Lana and Russell 1996).

From this understanding, some analogy can be made between the anti-protozoal effects of phospholipid analogues and the effects of lipids fed to ruminant animals. Although these are different compounds, they may have similar mechanisms of action. Although the mechanism of action is not completely clear, it appears that damage to the cellular membranes is an important facet of the toxicity of lipids upon protozoal cells.

1.7 Factors Affecting Protozoal Population in the Rumen

Many factors appear to influence protozoa numbers in the rumen. Attempts have been made to correlate ciliate numbers with diet and the availability of nutrients in various animals. Seasonal differences in food availability were shown to influence

population densities in wild ruminants (Giesecke and Van Gylswyk 1975). Diets containing high starch concentration are often reported to increase protozoal numbers (Walker and Nader 1970). Williams and Coleman (1992) stated that some species of ciliate disappear from individual sheep, and presumably cattle, for no apparent reason.

Competition between different species of ruminal protozoa can lead to exclusion of certain species. For instance, *Polyplastron* will eliminate *Epidinium*, *Eudiplodinium magi*, *Eudiplodinium spp*. and *Ostracdinium spp*. from a protozoal population (Dehority 2003). The reasons of this mutual exclusion have not been established. Dehority (2003) has speculated that the possible cause for some animals from the same herds to show divergent ciliate populations may be the presence of a factor in the saliva of some animals that kills or inactivates certain species rather than others.

Williams and Coleman (1992) reported research where specificity between the host species and their ciliates was observed; therefore, sheep, cattle, water buffalo, and others ruminants may show differences in type of ruminal fauna. These differences could be related to the feeding behavior of the host or to geographical distribution. It is also important to note that reports that a given species of protozoa occurs or does not occur in a given country only have validity when many animals have been examined from several different locations in that country.

Regarding the problem of variability on protozoa population, the first aspect that should be carefully addressed is the accurate determination of ciliate numbers. Firstly, the ruminal contents are not homogeneous; therefore, sub-samples from different sites should be taken so that a representative sample is collected. Some researchers remove all the ruminal content in order to obtain a better sample, but this procedure may be too laborious, especially when working with mature dairy cows. Secondly, phase separation must be accounted for. In most studies, the ciliates have been counted in the fluid phase.

This can lead to errors because there is a considerable attachment of protozoa to feed particles (Orpin 1984). Moreover, Dehority (1984) showed differences in counts, depending on whether one or more layers of cheesecloth were used to separate liquid and particulate phases.

Another aspect that should be observed is the number of ruminal sub-samples taken throughout the day that are used to examine ciliate numbers. Counts that are based on just one sampling may show high variability because of normal diurnal variation. Michalowski and Muszynski (1978) found in animals fed once a day that the endodiniomorphid protozoal population density declined 50% at 6h after feeding. The classical explanation for this behavior is that, immediately after feeding, the rumen fluid is diluted by water and saliva, thus reducing the protozoal population density (Williams and Coleman 1992). A short time after feeding, the protozoa, which are full of nutrients, begin to divide with the division rate reaching a maximum after 8h. After this time, cell division falls to almost zero. Animals fed twice a day or every 3h showed a similar pattern to those fed once a day, except that the cycles of peaks and troughs were shorter and reflected the feeding pattern (Michalowski and Muszynski 1978). These same authors reported that by fasting animals for a period of 48h reduced the protozoal population by 80-85%.

Froetschel et al. (1990), studying the effect of feeding frequency on protozoal population, reported that numbers increased from $2.18x10^6$/ml in steers fed once a day to $3.51x10^6$/ml in steers fed 12 times daily. According to Dehority (2003), holotrichs have a different pattern of growth than entodiniomorphids post feeding. This author reported that the entodiniomorphid population decreased, while holotrichs showed a marked increase. Dehority (2003) suggested that this increase in numbers after feeding could be chemotactic, assuming the cells detach and migrate toward a soluble energy source. This

suggestion was corroborated by the earlier work of Dehority and Mattos (1978). They observed the walls of the rumen and reticulum and noted that, in a number of animals, a thick protozoal mass appeared on the walls of the reticulum. This mass consisted of 75% holotrichs and 25% entodiniomorphids. They concluded that holotrichs sequestered on the walls of the reticulum migrate into the rumen contents at feeding time in a chemotactic response to incoming soluble nutrients. Another cause for the detachment of holotrichs from the reticulum walls could be the contractions associated with feeding (Dehority 2003). This author pointed out that the reduction in holotrich concentrations with frequent feeding might suggest that, as more frequent migrations from the reticulum walls occur, the more holotrichs are washed out of the rumen. Murphy et al. (1985) found similar responses of holotrich concentration to nutrient availability. When glucose was infused directly into the reticulum at the normal feeding time, a marked increase in holotrich numbers occurred. Infusion of water, saliva, NaCl or starch had no effect on holotrich concentrations.

Williams and Coleman (1992) report that experiments have shown seasonal variation where the protozoal population increases in the summer for no clear reason. Boyne et al. (1957) showed that there was up to a threefold variation in protozoal population density in the same animal kept on a constant diet under constant conditions.

Other aspect of diet composition can also alter ciliate type and population density. Dennis et al. (1983) reported an increase in ciliate densities as a result of starch addition to the diet. They found an increase in ciliate growth, mainly with entodinia. Usually, a forage-based diet provides a wider range of protozoal genera and species, while a concentrate-based diet gives rise to a protozoal population constituted mainly by entodiniomorphids (Williams and Coleman 1992). Grubb and Dehority (1975) showed an

abrupt raise in the protozoal population when the available energy of the diet was increased by switching a 100% forage diet to a 60% corn-40% forage diet.

However, the increase in protozoal numbers whith addition of concentrate is limited. Mackie et al. (1978) observed an increase of ciliate numbers when concentrate comprised from 10 to 60% of the diet, but protozoal numbers decreased when the level of concentrate was increased to 71%. This result suggests that pH may play a role modulating ciliate growth, even though not all data corroborates with this observation. Slyter et al. (1970) fed steers all concentrate diets ad libitum containing either 90% corn or 90% wheat. Only entodiniomorphids were present among the protozoal population; pH values were as low as 5.0-5.2. This situation implies that some genera like entodinia spp. may survive at very low ruminal pH.

Franzolin and Dehority (1996) studied the effect of prolonged high-concentrate diets, which resulted in a lowering on the concentration of ruminal protozoa. In general, feeding high-concentrate diets that result in a rumen pH below 6.0 result in a marked decrease in protozoal concentration.

Diets rich on soluble sugars are preferred by holotrichs (Valdez et al. 1977). These authors found that cattle fed sugar cane, molasses, and urea had a protozoal biomass comprised of 95% holotrich. The effect of urea is not clear it could have an indirect effect on the ciliates via the bacterial population. Onodera et al. (1977) suggested that ruminal protozoa are lacking in urease and urea is not a good source of N for their growth.

Regarding the effect of protein supplementation on protozoal numbers, as with urea, indirect effects cannot be ignored because there is little data from bacteria-free protozoa cultures in vitro. However, as discussed earlier, entodiniomorphids appear to be stimulated by insoluble proteins and holotrichs may show higher growth on soluble protein.

Dehority (2003) indicated that attention must be paid to the fact that, even when the concentration of protozoa is the same, effects on ruminal volume may mean the absolute number of protozoa are very different. Dehority (1978) fed two groups of sheep, either alfalfa or concentrate and found a statistical significant difference in protozoal concentrations. Nevertheless, when rumen volumes were determined on these animals and the total number of ciliates in the rumen was calculated, there was not different between the groups.

Moreover, in determination of protozoal population density, care must be taken in use of counting techniques such as dilution, preservation, staining, etc. Many different counting procedures are available in the literature (Demeyer and Van Nevel 1979; Ogimoto and Imai 1981; Clarke et al. 1982; Dehority 1984; Meyer and Mackie 1986; and Dehority 1993).

For all these reasons, comparisons between experiments involving protozoal counts obtained under different feeding regimes should be carefully interpreted.

1.8 Effects of Defaunation on Animal Performance

1.8.1 Growth

Protozoa grow more efficiently on diets containing proteins that are of low ruminal degradability due to their preference in engulfing feed particles (Ushida et al. 1986). Under these conditions, the absence of ciliates have shown to be positive, increasing the amount of rumen undegradable protein (RUP) and microbial protein flowing to the small intestines. In tropical conditions, ruminants are kept mainly on a pasture-based diet where protein is often the first limiting nutrient. Moreover, tropical pasture-based diets are mainly acetogenic and highly methanogenic. In such a scenario, defaunation may play an effective role, increasing protein flow, propionate formation and

decreasing methane formation in the rumen. Perhaps those effects explain why defaunation has increased animal growth on pasture-based diets (Bird and Leng 1978; Bird et al. 1979; Bird and Leng 1984; and Bird et al. 1994).

Nevertheless, much of the data available in literature concerning the effects of defaunation on animal performance are contradictory. This variation in response may be related to differences in animal requirements, especially the first limiting nutrient, age, physiological status, and diet. Furthermore, the defaunation procedure is an important factor influencing animal performance, mainly because most defaunation agents are toxic or are not specific and might also impair the bacterial population as well.

The increase in the microbial and feed N flow into the duodenum of defaunated animals is probably the clearest reason for enhancement of animal performance. However, reductions in digestibility may overcome the benefit of higher N flow.

1.8.2 Wool Production

Enhancement of wool production by defaunation in sheep has been frequently reported (Bird et al. 1979; Cottle 1988b; and Cottle 1988a). These results were attributed most often to an increase in flow of sulfur amino acids from rumen. These amino acids are often referred to as the first limitation to wool growth.

1.8.3 Milk Production

There are few data available in the literature concerning the effects of protozoa on milk production. Yang and Varga (1989) used four rumen cannulated cows in a switchback design to assess the effect of defaunation on milk production. Aerosol OT, dosed via rumen cannula, was used as the defaunating agent. The diets consisted of 45% grass silage and 55% concentrate. In that study, dry matter intake of faunated cows was adjusted to that of defaunated cows. Defaunation decreased milk production ($P < 0.10$)

from 27.6 to 24.6 Kg/d, milk protein (3.13 vs 3.00%) and lactose (4.98 vs 4.74%) in this experiment.

Recently, Hristov et al. (2004) used four cannulated cows in a crossover design to study the effect of using sodium laurate as the defaunating agent on fermentation and milk production. Compared to the control, protozoal population was reduced by 91%. Milk yield was not affected by treatment (28.8 vs 29.6 kg/d). Moreover, neither milk components (fat, protein and urea), nor N utilization efficiency were affected by sodium laurate. Both studies had only a limited number of animals. Consequently, it is difficult to determine precisely what effects defaunation would have on milk production.

1.9 Conclusions

Bacteria are present in protozoal vacuoles, where they undergo digestion by the ciliate (Coleman and Hall 1974), or in the protozoan cytoplasm surrounded by a polysaccharide capsule. Bacteria may also be found attached to the cuticle (Vogels et al. 1980). Thus, data from defaunation experiments should be carefully analyzed, and it must not be assumed that all characteristics attributed to ruminal ciliates are completely absent of bacterial action. However, recent experimental procedures have greatly reduced the chance for bias between protozoal and bacterial activity.

The most common overall effects of defaunation are summarized in Table 1. Although many faunation/defaunation experiments have been undertaken, some effects of defaunation remain debatable. In the light of current knowledge, the potential effects of defaunation on milk production cannot be predicted. Moreover, the effects of partial suppression of the protozoal population, rather than complete elimination of ciliates, have been poorly investigated. Indeed, studying the effect of partial or selective defaunation may contribute useful information, and may help to elucidate the roles protozoa play in the ruminal ecosystem and their effects on animal performance.

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1.11 Table

Table 1 – Effects most often seen with defaunation.

Reduced ruminal ammonia concentration
Increased bacterial population
Increased fungal population
Increased microbial protein synthesis and flow from rumen
Increased RUP flow from rumen
Reduced deamination
Reduced peptide's breakdown
Reduced methanogenesis
Decreased N excretion in urine
Increased N excretion in the feces
Reduced organic matter digestion
Reduced fiber digestion
Reduced total VFA production
Reduced the stability of ruminal pH

CHAPTER 2

EFFECTS OF LAURIC ACID ON RUMINAL PROTOZOA, FERMENTATION PATTERN AND MILK PRODUCTION IN DAIRY COWS

2.1 Introduction

Efficiency of utilizing dietary N for milk protein synthesis is low in dairy cows (Tamminga 1992; Castillo et al. 2000; Castillo et al. 2001; and Hristov et al. 2004). Therefore, improving N utilization is a major challenge in ruminant nutrition research. Overfeeding protein to dairy cows is uneconomical and results in excessive urinary N, the most environmentally labile form of excreted N (Varel et al. 1999).

Microbial protein synthesized in the rumen and RUP are the main sources of α-amino N supplied to ruminants. Ruminal ciliate protozoa have a negative effect on protein utilization in ruminants by reducing both microbial protein plus RUP flow from the rumen (Jouany 1996). The ciliates possess deaminase (Itabashi and Kandatsu 1975), peptidase (Newbold et al. 1989), and protease (Forsberg et al. 1984) activity; therefore they actively degrade dietary protein. Moreover, they briskly take up bacteria (Coleman 1975), and according to Wallace and McPherson (1987) protozoa are the main contributors to bacterial protein turnover in the rumen and increasing ruminal ammonia

concentration, which are central factors in determining the economic cost and environment impact of ruminant production (Hristov et al. 2004).

Defaunation appears to reduce ruminal pH stability (Veira et al. 1983), fiber digestion and total VFA production (Jouany et al. 1988). This perhaps explains the inconsistent animal performance reported in defaunation studies. However, partial suppression of protozoal populations has received little attention.

Most defaunation techniques are harmful to animals and are not applicable under practical feeding conditions. Medium chain fatty acids have been shown to have a potent anti-protozoal effect (Newbold and Chamberlain 1988; Matsumoto et al. 1991; Dohme et al. 2000; Machmuller et al. 2002; Soliva et al. 2003; Faciola et al. 2004; and Hristov et al. 2004). Furthermore, they can be used routinely in farm operations and their use is allowed in European countries that prohibit other feed additives such as antibiotics.

The current state of knowledge regarding partial suppression of protozoal populations in the rumen apparently does not allow for conclusive understanding of its effects in milk production and efficiency of N utilization. We hypothesized that partial suppression of protozoal population would improve N utilization without being harmful to carbohydrate fermentation, thus enhancing animal performance. Hence, the aims of this study were: (1) To evaluate lauric acid as a practical defaunating agent, (2) to assess the effects of partial defaunation on fermentation patterns and milk production in dairy cows.

2.2 Materials and Methods

2.2.1 Experiment 1

2.2.2 Experimental Design, Diets, and Animal Management

Six multiparous Holstein cows (one dry), averaging 660 (SD 46) kg of BW, parity 3.5 (SD 1), 102 (SD 23) DIM, and 40.4 (SD 6.2) kg/d of milk; fitted with ruminal cannulae were blocked into groups of 3 by DIM to give 2 blocks in a trial of randomized complete block design.

Care and handling of all experimental animals, including ruminal cannulation was conducted under protocols approved by the University of Wisconsin Institutional Animal Care and Use Committee. Prior to starting the experimental phase of the trial, all cows were fed the same diet (control, without LA) for a 2-wk covariate period, and dry matter intake and protozoal counts were determined for use in statistical analysis. Cows within blocks of 3 were then randomly assigned to one of the 3 diets and fed only that diet during the remaining study.

The treatments were: 1) Control, 2) 160g/d of lauric acid (LA; KIC chemicals Inc., 99% purity), or 3) 160g/d of sodium laurate (NaLA) dissolved in 1,600 ml of water as described by Hristov et al. (2004). Both LA and NaLA were given in a single dose into the rumen via cannulae before feeding. The TMR contained (DM basis): 15% alfalfa silage, 40% corn silage, 30% rolled high moisture shelled corn (HMSC), 14% solvent-extracted soybean meal (SSBM), 16.6% CP and 29% NDF (Table 1).

Cows were housed in tie stalls bedded with straw and had free access to water throughout the trial. Cows were milked twice daily at 5:00 a.m. and 5:00 p.m. Individual milk yields were recorded at each milking.

All cows were injected with bST (500 mg of Posilac; Monsanto, St. Louis, MO) beginning on d-1 of the trial and at 14-d intervals throughout the trial. Cows were fed the TMR for ad libitum intake once a day and orts were collected and weighed daily at 5 p.m. The feeding rate was adjusted daily to yield orts of about 10% of intake.

Weekly composites of the TMR, orts, alfalfa silage, corn silage, and HMSC were collected from daily samples of about 0.5 kg and stored at -20°C. Weekly samples of the 48% CP solvent-extracted soybean meal were stored at 21 to 24°C. Proportions of each ration ingredient on an as-fed basis were adjusted weekly based on total N content and DM determined by drying weekly composites at 60°C (48 h) for alfalfa silage, corn silage, and HMSC and at 105°C (AOAC 1980) for SSBM; salt, sodium bicarbonate, dicalcium phosphate, mineral and vitamin supplements were assumed to have 100% DM. Intake of DM was computed based on the 60°C DM values for TMR and orts. After drying, major dietary ingredients and TMR were ground through a 1-mm screen (Wiley mill; Arthur H. Thomas, Philadelphia, PA) and then analyzed for DM at 105°C, ash and OM (AOAC 1980), total N by combustion assay (Leco 2000; Leco Instruments, Inc., St. Joseph, MI) and sequentially for NDF and ADF using heat stable amylase (Van Soest et al. 1991) and Na_2SO_3 (Hintz et al. 1995) during the NDF step. Composition data in Table 1 were from analysis of ingredient composites. At the end of the trial, weekly composites of alfalfa silage and corn silage were thawed, water extracts were prepared, deproteinized, and then analyzed for NPN (Muck 1987) using a combustion N assay (Mitsubishi TN-05 Nitrogen Analyzer; Mitsubishi Chemical Corp., Tokyo).

About 100-200 ml of digesta were collected from 4 different sites of the rumen of cows at 0 (just before feeding), 1, 2, 4, 8, 12, 18 and 24 h after the feeding on the last day of d-8 and d-16 of the experimental phase, strained through two layers of cheesecloth, and pH measured immediately by glass electrode. One sample from each cow was preserved

at each time point by adding 0.2 ml of 50% (vol/vol) H_2SO_4 to 10-ml of strained ruminal fluid and storing samples at –20°C. Just prior to analysis, samples were thawed and centrifuged (15,300 x g for 20 min at 4°C). Flow-injection analyses (Lachat Quick-Chem) were applied to supernatants to determine ammonia using a phenol-hypochlorite method (Lachat Method 18-107-06-1-A) and total AA using a fluorimetric procedure based on reaction with o-phthalaldehyde (Roth 1971). Leucine was the standard in the o-phthalaldehyde assay and total AA are reported in leucine equivalents. Samples also were thawed and centrifuged (28,000 x g for 30 min at 4°C) for determination of individual and total ruminal VFA using a modification of the GLC method for free fatty acids described in Supelco Bulletin 855B (Supelco Inc., Supelco Park, Bellefonte, PA) with flame-ionization detection. Standards or supernatants (1 μl) were injected onto a ZB-FFAP capillary column (30mm x 0.53 mm x 1.0 mm; no. 7HK-G009-22; Phenomenex Inc., Torrance, CA) with helium carrier gas at 100 KPa and a flow rate of 20 ml/min. Column oven temperature was 100°C at injection; after 2 min., the temperature was ramped up to 130°C at 10°C/min. Injector and detector temperatures were 230°C and 250°C. Response areas from standards were used to compute VFA concentrations in ruminal samples. The method did not resolve isovalerate and 2-methylbutyrate. Individual VFA are reported in concentration units rather than as molar proportions.

Total protozoa numbers in rumen contents were determined as described by Dehority (1984), and Dehority (1993). Briefly, 10 ml of strained rumen fluid were taken from cannulated cows at eight time points after feeding (0, 1, 2, 4, 8, 12, 18, and 24h) and mixed with 10 ml of 50% formalin (18.5% vol/wt formaldehyde). Two drops of brilliant green dye were added to 1-ml aliquots and allowed to stand overnight. After staining, 9 ml of 30% glycerol solution was added, and the diluted samples were pipetted into a

Sedgewick-Rafter counting chamber (1-cm^3 volume). Further dilutions were made with 30% glycerol when needed. Protozoa were counted at a magnification of 100×.

2.2.3 Experiment 2

2.2.4 Experimental Design, Diets, and Animal Management

Thirty-two multiparous Holstein cows (8 with ruminal cannulae) averaging parity 2.6 (SD 1), 633 (SD 52) kg of BW, 152 (SD 72) DIM, and 43.1 (SD 11) kg milk/d, and twenty primiparous Holstein cows averaging 566 (SD 39) kg of BW, 123 (SD 42) DIM, and 40.5 (SD 2.6) kg milk/d were blocked into groups of 4 by DIM to give 8 multiparous blocks (2 of cows with ruminal cannulae) and 5 primiparous blocks in a trial of randomized complete block design.

Care and handling of all experimental animals, including ruminal cannulation was conducted under protocols approved by the University of Wisconsin Institutional Animal Care and Use Committee. Prior to starting the experimental phase of the trial, all cows were fed the same diet (control) for a 2-wk covariate period and production of milk and milk components was determined for use in statistical analysis. Cows within blocks of 4 were then randomly assigned to one of the 4 diets and fed only that diet during the remaining 8 weeks of the study.

The four experimental diets were similar except that some of the finely ground dry corn was replaced with either finely ground dry corn or finely ground dry corn plus lauric acid (8% LA and 92% corn, DM basis) in stepwise increments from 0 to 12.16% of dietary DM (Table 2). This product contained 99% lauric acid and was acquired from KIC chemicals, Inc. The cost of this product was U$ 3.04/kg.

Cows were housed in tie stalls bedded with straw and had free access to water throughout the trial. Cows were milked twice daily at 5:00 a.m. and 5:00 p.m. Individual milk yields were recorded at each milking. Milk samples were collected at two consecutive milkings midway through wk-2, 4, 6, and 8 of the experimental phase and analyzed for fat, protein, lactose, and SNF by infrared methods (Foss Milkoscan 4000, Foss North America, Eden Prairie, MN; AgSource, Verona, WI). Milk samples were deproteinized (Shahani and Sommer 1951) and analyzed for milk urea N (MUN) by an automated colorimetric assay (Broderick and Clayton 1997) adapted to a flow-injection analyzer (Lachat Quick-Chem 8000 FIA; Zellweger Analytical, Milwaukee, WI). Concentrations and yields of fat, protein, lactose, and SNF, and MUN concentrations, were computed as the weighted means from p.m. and a.m. milk yields on each test day. Yields of 3.5% FCM also were computed (Sklan et al. 1992). Body weights were measured on 3 consecutive d at the start and end of the 8-wk experimental phase to compute BW change.

All cows were injected with bST (500 mg of Posilac; Monsanto, St. Louis, MO) beginning on d-1 of the trial and at 14-d intervals throughout the trial. Cows were fed a TMR for ad libitum intake once a day and orts were collected and weighed daily at 5 p.m. The feeding rate was adjusted daily to yield orts of about 10% of intake. Diets were adjusted weekly to reflect changes in DM and CP of forages and concentrate mixtures by drying weekly composites of each ingredient overnight in an oven at 105°C and analyzing for total N.

Weekly composites of the TMR, orts, alfalfa silage, corn silage, and HMSC were collected from daily samples of about 0.5 kg and stored at -20°C. Weekly samples of the corn grain, corn grain plus LA, and SSBM were stored at 21 to 24°C. Proportions of each ration ingredient on an as-fed basis were adjusted weekly based on DM determined by

drying weekly composites at 60°C (48 h) for alfalfa silage, corn silage, and HMSC and at 105°C (AOAC 1980) for corn grain, corn grain plus LA, and SSBM; salt, sodium bicarbonate, dicalcium phosphate, mineral and vitamin supplements were assumed to have 100% DM. Intake of DM was computed based on the 60°C DM values for TMR and orts. After drying, major dietary ingredients and TMR were ground through a 1-mm screen (Wiley mill; Arthur H. Thomas, Philadelphia, PA) and then analyzed for DM at 105°C, ash and OM (AOAC 1980), total N by combustion assay (Leco 2000; Leco Instruments, Inc., St. Joseph, MI), sequentially for NDF and ADF using heat stable amylase (Van Soest et al. 1991) and Na_2SO_3 (Hintz et al. 1995) during the NDF step. Data on composition in Table 2 were from analysis of ingredients composites. At the end of the trial, weekly composites of alfalfa silage and corn silage were thawed, water extracts were prepared, deproteinized, and then analyzed for NPN (Muck 1987) using a combustion N assay (Mitsubishi TN-05 Nitrogen Analyzer; Mitsubishi Chemical Corp., Tokyo).

At the end of wk-4 and wk-8 of the experimental phase, urine samples were collected from all cows at about 6 and 18 h after feeding. Fresh urine samples were acidified by diluting 1 volume of urine with 4 volumes of 0.072 N H_2SO_4 and storing at –20°C until analyzed. At the end of the trial, all urine samples were thawed at room temperature, filtered through a Whatman no. 1 filter paper. Filtrates were analyzed for creatinine using a picric acid assay (Oser 1965) adapted to the flow-injection analyzer (Lachat Quick-Chem), for total N (Mitsubishi Nitrogen Analyzer), for allantoin using the method of Vogels and Van Der Grift (1970) adapted to a 96-well plate reader, for uric acid using a commercial kit (No. 683-100P, Sigma Chem. Co., St. Louis, MO), and for urea with the colorimetric method used for MUN. Daily urine volume and excretion of urea N, total N, and purine derivatives (PD; allantoin plus uric acid) were estimated from

mean urinary concentrations assuming a creatinine excretion rate of 29 mg/kg of BW (Valadares et al. 1999).

About 100-200 ml of digesta were collected from 4 different sites of the rumen of ruminally cannulated cows at 0 (just before feeding), 1, 2, 4, 8, 12, 18 and 24 h after the feeding on the last day of wk-4 and wk-8 of the experimental phase, strained through two layers of cheesecloth, and pH measured immediately by glass electrode. One sample from each cow was preserved at each time point by adding 0.2 ml of 50% (vol/vol) H_2SO_4 to 10-ml of strained ruminal fluid and storing samples at –20°C. Just prior to analysis, samples were thawed and centrifuged (15,300 x g for 20 min at 4°C). Flow-injection analyses (Lachat Quick-Chem) were applied to supernatants to determine ammonia using a phenol-hypochlorite method (Lachat Method 18-107-06-1-A) and total AA using a fluorimetric procedure based on reaction with o-phthalaldehyde (Roth 1971). Leucine was the standard in the o-phthalaldehyde assay, and total AA are reported in leucine equivalents. Samples also were thawed and centrifuged (28,000 x g for 30 min at 4°C) for determination of individual and total ruminal VFA using a modification of the GLC method for free fatty acids described in Supelco Bulletin 855B (Supelco Inc., Supelco Park, Bellefonte, PA) with flame-ionization detection. Standards or supernatants (1 μl) were injected onto a ZB-FFAP capillary column (30mm x 0.53 mm x 1.0 mm; no. 7HK-G009-22; Phenomenex Inc., Torrance, CA) with helium carrier gas at 100 KPa and a flow rate of 20 ml/min. Column oven temperature was 100°C at injection; after 2 min., the temperature was ramped up to 130°C at 10°C/min. Injector and detector temperatures were 230°C and 250°C. Response areas from standards were used to compute VFA concentrations in ruminal samples. The method did not resolve isovalerate and 2-methylbutyrate. Individual VFA are reported in concentration units rather than as molar proportions.

Total protozoa numbers in rumen contents were determined as was done in the first trial.

2.2.5 Statistical Analyses

Data were analyzed using the Proc Mixed procedure of SAS (version 8 SAS (1999); SAS Institute Inc., Cary, NC). A single mean value was computed for each cow over the complete experimental phase of the trial for DMI, milk production, and protozoal counts. To these variables, a model that included the covariate mean for each cow and the dietary treatment was fitted. The following model was used for ruminal variables for which there were repeated measurements over time (pH, ammonia, total free AA and VFA): $Y_{klm} = \mu + Vk_{(l)} + T_l + Z_m + ZT_{ml} + E_{klm}$, where Y_{klm} = dependant variable, μ = overall mean, $Vk_{(l)}$ = effect of cow k within treatment l, T_l = effect of treatment l, Z_m = effect of time m, ZT_{ml} = interaction between time m and treatment l, and E_{klm} = residual error. The spatial covariance structure SP (POW) was used for estimating covariances and the subject of the repeated measurements was defined as cow (treatment). All terms were considered fixed, except for $Vk_{(l)}$, and E_{klm}, which were considered random. Significance in each trial was declared at $P \leq 0.10$, and separation of least squares means was conducted at $\alpha = 0.10$ using pdiff in the least squares means statement.

2.3 Results and Discussion

2.3.1 Trial 1

Lauric acid given intraruminally at 160g/cow/day did not reduce DMI but sodium laurate did (Table 3). Hristov et al. (2004) found no reduction in DMI when 240g of sodium laurate was infused daily divided into two equal doses of 120 g. The authors also reported that the cows went off feed when 320g of sodium laurate were infused daily,

even when it was divided into two doses. Dosing NaLA twice a day appeared to be less detrimental to DMI and using a single dose of 160 g may be the explanation for the present results.

The mechanisms by which supplemental fat depresses feed intake are not clear but may involve effects on ruminal fermentation and gut motility, palatability, release of gut hormones, and oxidation of fat in the liver (Allen 2000). In addition, the explanation why NaLA reduced DMI while LA did not could be related to the fact that NaLA dissolved in the ruminal fluid phase, while LA became associated with the particulate phase. Indeed, NaLA could flow out of the rumen faster and be more easily absorbed, stimulating the release of CCK, which is related to feed intake depression (Grovum 1981).

Excessive degradation of forage proteins usually is wasteful due to excessive ammonia formation in the rumen (Broderick et al. 2004). Feeding studies have confirmed that alfalfa protein often is poorly utilized by lactating dairy cows (Broderick 1985; Broderick 1995b). In the present trial, ruminal ammonia decreased sharply with both treatments. A reduction in ruminal ammonia concentration is often reported in defaunation studies (Ushida et al. 1986; Williams and Coleman 1992; and Jouany 1996); this is mainly associated with the ability of protozoa to ingest and degrade bacterial proteins (Broderick et al. 1991) and protozoal deaminase activity (Wallace et al. 1987). The cows on the control diets showed low ruminal ammonia concentration, even thought both LA and NaLA were effective in lowering ruminal ammonia concentration. Ruminal ammonia concentration observed in the LA treatment was even lower than the minimum concentration of 5 mg/dl (2.94 mM) proposed by Satter and Slyter (1974), which may have impaired ruminal function, especially cell-wall carbohydrates fermentation. Moreover, beyond reducing recycling, defaunation generally increases microbial growth

(Jouany 1996), which uses a large proportion of ammonia N (Hristov and Broderick 1996).

In this study, both treatments reduced the concentration of ruminal total free amino acids. Most of the NPN in alfalfa silage is present as free AA and small peptides (Broderick 1995a). Broderick et al. (1988) indicated that free peptides originating from dietary protein degradation accumulate in the rumen, becoming available for protozoal attack. In the presence of ruminal protozoa, due to active protozoal peptidases (Newbold et al. 1989) and proteases (Naga and el-Shazly 1968; Coleman 1983; Forsberg et al. 1984; and Nagasawa et al. 1994), dietary proteins and peptides are more susceptible to cleavage, which will release free amino acids into the medium. Moreover, Coleman (1975) proposed that ciliates utilize only about half of their ingested N; the rest is expelled as short-chain peptides and free amino acids. This may explain why free amino acid concentration in the rumen decrease with partial defaunation.

Perez Alba et al. (1997) reported that a higher ruminal pH was detected when calcium soap was fed to ewes. Hristov et al. (2004) also reported an increase on ruminal pH after dosing with NaLA. These workers also found an abnormal ruminal pH increase when more than 320g of NaLA was infused daily. Defaunation studies often report that a lack of ciliates could lead to ruminal pH instability (Veira et al. 1983) due to the ability of protozoa to slow the fermentation of sugars and non-structural carbohydrates by reducing the availability of those carbohydrates to bacterial fermentation. Ruminal pH is a critical determinant for rumen function as cellulolytic bacteria fail to grow below a pH value of approximately 6.0.

Lipids per se are unlikely to alter ruminal pH. Most of the effects on ruminal pH reported when fats were added to the diet were attributed to the reduction of structural carbohydrates digestion and protozoa elimination (Jenkins 1993). Moreover, ruminal pH

usually is decreased by feeding lipids. The reasons why NaLA increased ruminal pH were not clear and perhaps were related to its buffering capacity or another physiological characteristic of NaLA.

Most literature has reported a decrease in total VFA production as a consequence of defaunation. This has been usually attributed to the reduction in carbohydrate fermentation, especially cell-wall carbohydrates. Moreover, the role protozoa play in maintaining pH stability by slowly fermenting sugars and starch and metabolizing lactic acid also may have an impact on total VFA production. There was no difference in total ruminal VFA concentration for both treatments. This may be due to the high digestibility of the diet, and depression of structural carbohydrates digestion, if it happened, had little apparent influence on overall fermentation pattern. The effect of defaunation on the proportions of VFA is variable, although the results of experiments frequently have shown an increase in molar proportion of propionate at the expense of butyrate and acetate. Acetate and butyrate are produced by ruminal ciliates during carbohydrate fermentation (Williams and Coleman 1997). Therefore, this may explain the reduction in the molar proportion of these acids after defaunation.

Jouany et al. (1988) suggested that defaunation leads to the formation of butyrate via acetyl-CoA, as well as to a stimulation of the conversion of acetate into butyrate. According to the same authors, this second pathway could explain the shift in VFA towards production of butyrate that is sometimes observed in defaunated animals given diets rich in soluble carbohydrates. However, in this trial the molar proportion of butyrate was reduced by NaLA, corroborating other studies that reported the same pattern (Kayouli et al. 1984; Ushida et al. 1986; Hsu et al. 1991a; Hsu et al. 1991b; Dohme et al. 1999; Koenig et al. 2000; Nelson et al. 2001; and Ueda et al. 2003).

Veira (1986) suggested that because of the inconsistency of the effects of defaunation on VFA proportions, we should not assume that ciliates are directly responsible for the observed differences. This author suggested that changes in the bacterial population probably would play a role in the pattern of VFA production. Some of the experiments that have shown an increase in total VFA production have been attributed to the increase in bacterial population in the rumen, which is commonly observed in defaunation studies.

Changes reported in VFA proportion as a result of feeding lipids, are similar to those found in defaunation studies; usually, the molar proportion of acetate decreases and propionate increases. This fermentation pattern was observed by Jenkins (1990); Bateman and Jenkins (1998); Nelson et al. (2001); and Ruppert et al. (2003).

The main result of this study was the significant reduction of protozoal populations observed when both LA and NaLA were infused daily into the rumen. Others studies have reported the inhibitory activity of lauric acid on ruminal protozoa (Matsumoto et al. 1991; Dohme et al. 2000; Hristov et al. 2000; Machmuller et al. 2001; Machmuller et al. 2002; Soliva et al. 2003; and Hristov et al. 2004). It is well accepted that lipids, in general, possess an anti-protozoal effect. However, the mechanisms by which this occurs are still unclear. Jenkins (1993) has suggested that anti-microbial effects of lipids in the rumen may have similarities to cytotoxic effects of fatty acids on biological membrane functions, such as oxidative phosphorylation.

Perturbation of ether-lipid metabolism (Lux et al. 1996), inhibition of an enzyme (alkyl-specific acyl-Co-A) involved in lipid-remodeling (Lux et al. 2000), and inhibition of phosphatidylcholine (PC) biosynthesis by preventing the incorporation of methionine into PC, thereby blocking de novo synthesis (Lira et al. 2001), have been suggested as possible mechanisms for lipid toxicity to protozoa.

The first site of interaction of phospholipid analogues with protozoa is the cell membrane (Croft et al. 2003). Ultra-structural analysis of effects of some phospholipid analogues against protozoal cells showed extensive membrane blebbing, that may lead to membrane dysfunction and ultimately cell death (Santa-Rita et al. 2000). Interferences with carrier proteins could also cause depletion of essential nutrients and, thus, lead to cell death or at least contribute to growth retardation. Furthermore, Na^+/ K^+-ATPase, another important enzyme in signal transduction, is inhibited by phospholipid analogues (Berkovic et al. 1992). A similar effect on K^+ depletion was observed in ruminal bacteria when exposed to ionophores (Lana and Russell 1996).

2.3.2 Trial 2

There was no effect on DMI in this trial so it appeared that inclusion of LA appeared not to affect the palatability of the diet. Matsumoto et al. (1991) reported that the addition of 25g/d of free fatty acids to the diets (5% of DMI) resulted in a decrease in appetite of dairy goats; however, fatty acid derivatives such as salts and triglycerides did not decrease appetite.

Ruminal pH and ruminal metabolites, such as NH_3, and total free amino acids were not different among treatments (Tables 4 and 5). It appears that the doses of LA, used when given in the TMR, were not high enough to reach a concentration of LA within the rumen to promote a reduction on ruminal protozoal population at the magnitude expected based on our previous study (Faciola et al. 2004).

Milk production was not statistically different among treatments. In all of them, a numerical reduction on milk production was observed; nevertheless, the reduction was less severe on the D treatment, and it may have been a consequence of higher doses of LA. This may be an indication of improvement on nutrient utilization for milk synthesis.

Much caution was taken in order to avoid errors in the protozoal counts. However, as pointed out by Williams and Coleman (1992), and Dehority (2003), several things may interfere with the determination of protozoal numbers in the rumen. In the second trial, the protozoal population was reduced by 25% and 30% in treatments C and D respectively. Compared to infusing LA directly into the rumen, feeding the cows the LA in the TMR did not achieve the same level of inhibition of the ciliate population. The feeding pattern of the cows may have had an impact on the response. Friggens et al. (1998) observed that cows eating a diet with 50% concentrate (DM basis) made about 30 visits to the feed bank per day. It appears that in our second trial, the concentration of LA within the rumen may not have reached the same concentration that was obtained in the first, which was about 0.2% of ruminal content (assuming a ruminal weight of 80 kg). It seems that, if a certain level of protozoal suppression is not achieved, the remaining protozoa are able to reestablish the previous population by replication, which takes less than 12h (Williams and Withers 1993; and Dehority 1998).

Matsumoto et al. (1991) did not observe a difference in the *Epidinium caudatum* population when 25g of LA was fed daily for 5 days to goats. This amount represented 5% of DMI or about 1g/kg of BW. Furthermore, coconut oil gave the same results when fed at 30 g/d for 10 days. Although the *Epidinium caudatum* population was unaffected by either LA or coconut oil, all other ruminal protozoal species were eliminated from the rumen after both treatments.

Machmuller et al. (2001), feeding 58g/d of coconut oil to sheep within two meals per day (5.95% of DMI), found no difference in the population of Entodiniomorphids, which usually comprises about 90% of ruminal protozoal population (Franzolin and Dehority 1996; and Hristov et al. 2001). However, Machmuller et al. (2001) also found a significant reduction in the population of Holotrichs. In the in vivo phase of this study,

the total ruminal protozoal population was reduced by about 50% with the coconut oil treatment. Machmuller et al. (2001) also conducted two in vitro studies, using an intensive diet (ID; 32%NDF and 22.5% CP) and an extensive diet (ED; 50%NDF and 14% CP). Coconut oil (about 5% of DM) reduced the protozoal population by 95% with the ID but only by 50% with the ED. In addition, lauric acid (5% DM) completely eliminated ruminal protozoa from the medium. We must take into consideration that the concentrations used in the work of Machmuller et al. (2001) were much higher than those used in our study. Moreover, the reasons why Entodiniomorphids were not affected in the in vivo trial and why the effects of LA on protozoal population were lower with the ED diet are not clear.

Ueda et al. (2003) also found an interaction between the basal diet (high forage versus high concentrate) and oil supplementation for protozoal counts. They did not observe a reduction in protozoal numbers when a high forage diet was given; instead, oil supplementation was effective in reducing the protozoal population when a high concentrate-base diet was fed. These findings are in agreement with the in vitro study conducted by Machmuller et al. (2001). It appears that, with high-forage diets, the anti-protozoal effects of different agents are less severe and the reasons for this are not clear.

Ankrah et al. (1990) used four steers to evaluate two defaunation protocols as follow: (A) two days of dosing with 40g/d of dioctyl sulfosuccinate (DSS) via rumen cannulae; and (B) ten days of feeding 40g/d of DSS. Ten days post-dosing with DSS (treatment A) protozoa were absent from the rumen of three steers, but one steer had a small ruminal protozoal concentration, which was probably due to incomplete defaunation during dosing. Although there was a trend for lower protozoal concentrations for steers fed 40 g of DSS daily (treatment B), compared to steers prior to dosing, mean protozoal concentration did not differ; only one steer remained free of protozoa while

being fed 40 g of DSS daily for ten days. These results corroborate our findings and show that the anti-protozoal effects differ between infusing and feeding such agents.

2.4 Conclusion

Both lauric acid and sodium laurate showed high anti-protozoal activity when a single dose of 160 g/d via ruminal canulae, reducing the ciliate population within the rumen by 90% in a few days of treatment. Lauric acid reduced ruminal ammonia concentration by 60% without reducing DMI. Both agents reduced ruminal free amino acid concentration. Lauric acid did not affect ruminal pH, or reduce total ruminal VFA concentration. Under the condition of the first trial, Lauric Acid was shown to be a potent defaunation agent, obviating the need to use Na-laurate, which is more costly. Lauric acid fed in the TMR at 80, 160, or 240 g/d did not reduce DMI, and did not affect ruminal pH, ruminal parameters, nor milk production. Lauric acid fed at 160 and 240 g/d in the TMR of Holstein dairy cows reduced the ruminal protozoal population by only 25 and 30% respectively, showing that these levels in the diet were not sufficient to achieve a concentration within the rumen that promoted the anti-protozoal effect of lauric acid.

Further studies are necessary to determine the concentration of lauric acid in the TMR that is necessary to promote high protozoal suppression and to identify a practical way to obtain that level. In addition, the degree of protozoal suppression, which may be beneficial for nutrient utilization in the dairy cow, still unknown.

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2.6. Tables

Table 1 Composition of the diet from experiment 1.

Item	Diet
	(% of DM)
Ingredients	
Alfalfa silage	15.0
Corn silage	40.0
Rolled HMSC	29.5
48%SSBM	14.0
Sodium bicarbonate	0.5
Limestone	0.3
Salt	0.3
Dicalcium phosphate	0.2
Vit. & Trace min.	0.2
Nutrient content of diet	
CP	16.6
NDF	29

Table 2 Composition of the diet from experiment 2.

Ingredients	Diets (% DM)			
	A	B	C	D
Alfalfa Silage	28.81	28.81	28.81	28.81
Corn Silage	35.72	35.72	35.72	35.72
Rolled HMSC	14.10	14.10	14.10	14.10
48% Soybean meal	7.59	7.59	7.59	7.59
Sodium bicarbonate	0.75	0.75	0.75	0.75
Limestone	0.36	0.36	0.36	0.36
Salt	0.19	0.19	0.19	0.19
Dicalcium phosphate	0.24	0.24	0.24	0.24
Vitamins & Minerals	0.08	0.08	0.08	0.08
Corn grain ground dry	12.16	8.11	4.05	0.00
Premix[1]	0.00	4.05	8.11	12.16
Composition				
Crude Protein	15.50	15.70	15.90	16.10
NDF	28.7	28.8	28.8	28.8
ADF	17.4	17.5	17.5	17.6

[1] premix made of 92% corn grain ground dry and 8% lauric acid

Table 3. Effects of Lauric Acid or Sodium Laurate on dry matter intake and ruminal parameters from experiment 1.

Item	Control	LA	NaLA	SEM	P>F
DMI, kg/d	25.3[a]	23.8[a]	21.7[b]	0.5	0.07
Ammonia, mM	6.6[a]	2.6[c]	4.6[b]	0.5	<0.01
Total free AA, mM	10.4[a]	6.6[b]	3.9[c]	0.8	<0.01
pH	6.25[b]	6.38[b]	6.69[a]	0.09	0.08
Total VFA, mM	75.6	71.6	63.6	13.7	0.83
Acetate, mM	42.6	38.0	38.6	7.6	0.90
Propionate, mM	18.7	20.0	15.4	5.13	0.82
Butyrate, mM	10.0[a]	8.0[ab]	5.7[b]	0.8	0.09
Isobutyrate, mM	0.98	0.91	0.92	0.10	0.89
Isovalerate, mM	1.53	1.92	1.71	0.13	0.18
Valerate, mM	1.76	2.66	1.23	0.37	0.15
Protozoa, x 10^6 cells/ml	5.90[a]	0.37[b]	0.51[b]	0.34	<0.01

[a,b,c] Means in rows without common superscripts are different ($P < 0.10$).

Table 4 Dry matter intake, protozoal count, and milk production from experiment 2.

Item	A	B	C	D	SEM	P>F
DMI, kg/d	26.6	25.5	25.3	25.0	0.55	0.10
Milk, kg/d	35.27	36.10	35.78	36.47	0.78	0.73
Protozoa, x 10^6 cells/ml	5.05[a]	5.16[a]	3.81[b]	3.43[b]	3.77	0.05

[a,b] Means in rows without common superscripts are different ($P < 0.10$).

Table 5 Ruminal parameters from experiment 2.

Item	A	B	C	D	SEM	P>F
Ammonia, mM	6.11	6.24	6.57	7.51	0.61	0.40
Total free AA, mM	11.56	9.52	12.43	9.43	1.03	0.23
pH	6.56	6.60	6.51	6.40	0.06	0.27

2.7. Figures

Figure 1. Effects of Lauric Acid or Sodium Laurate on protozoal counts from experiment 1.

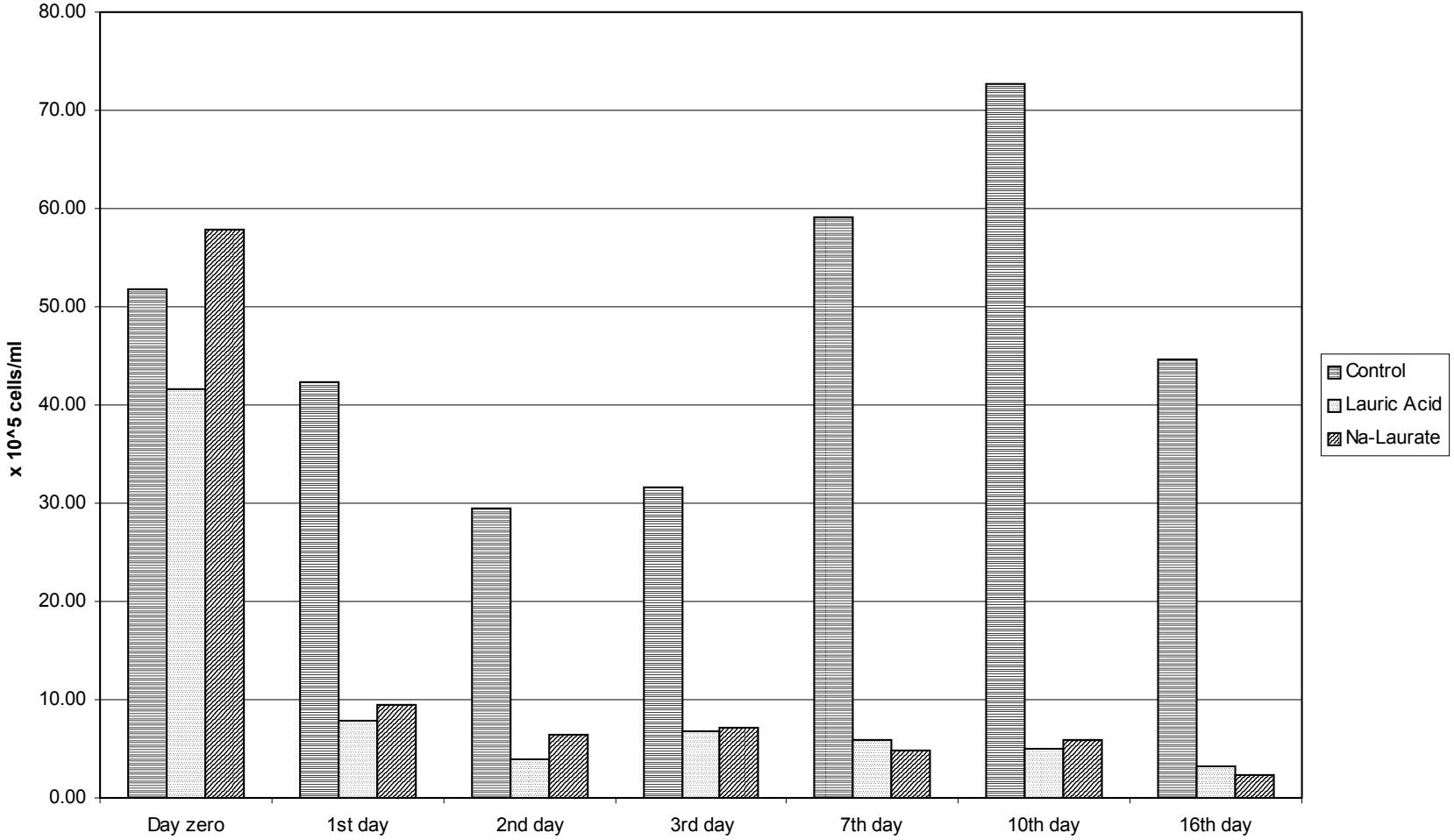

Figure 2. Effects of Lauric Acid or Sodium Laurate on protozoal counts after starts infusion from experiment 1.

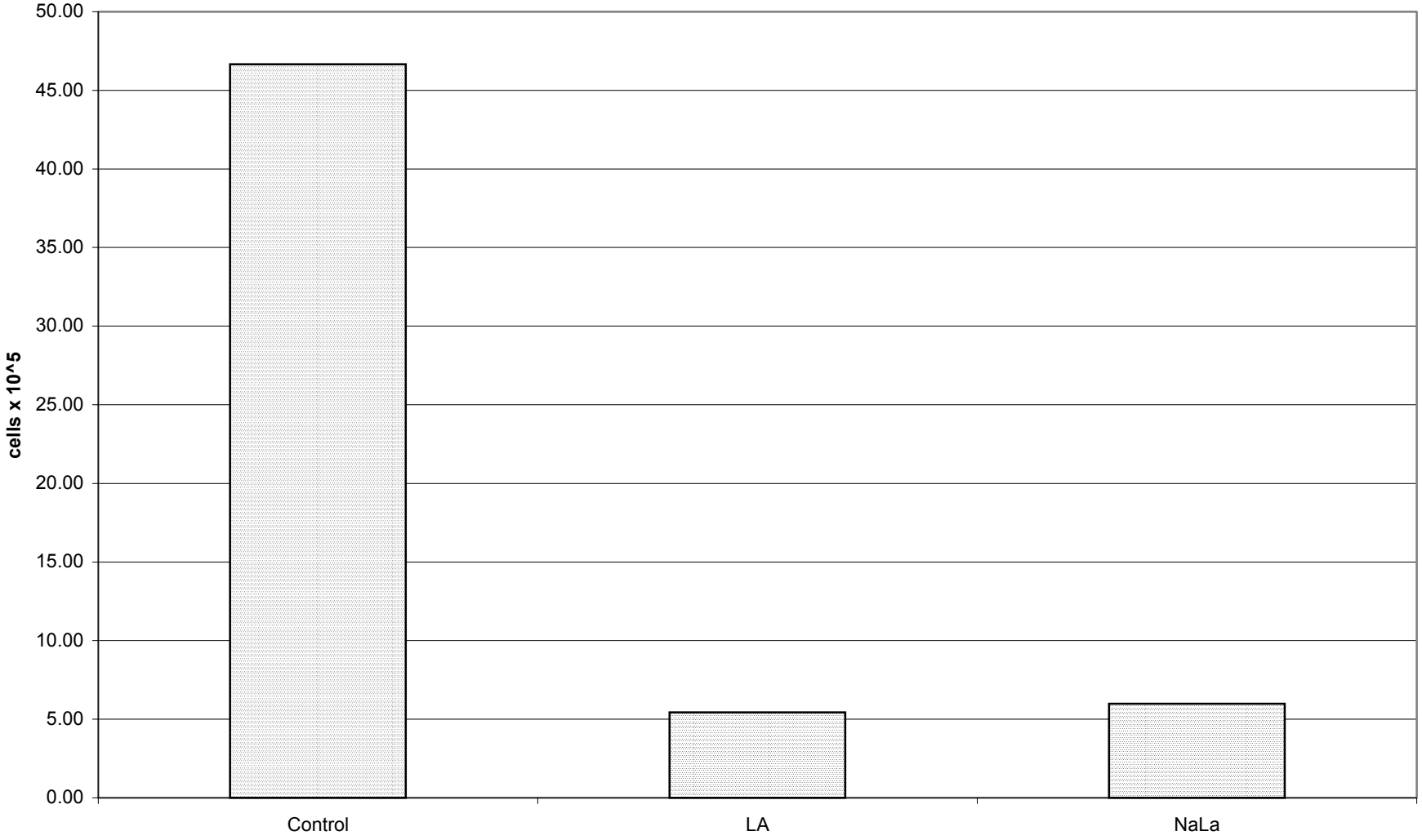

Figure 3. Effects of Lauric Acid on protozoal counts from experiment 2.

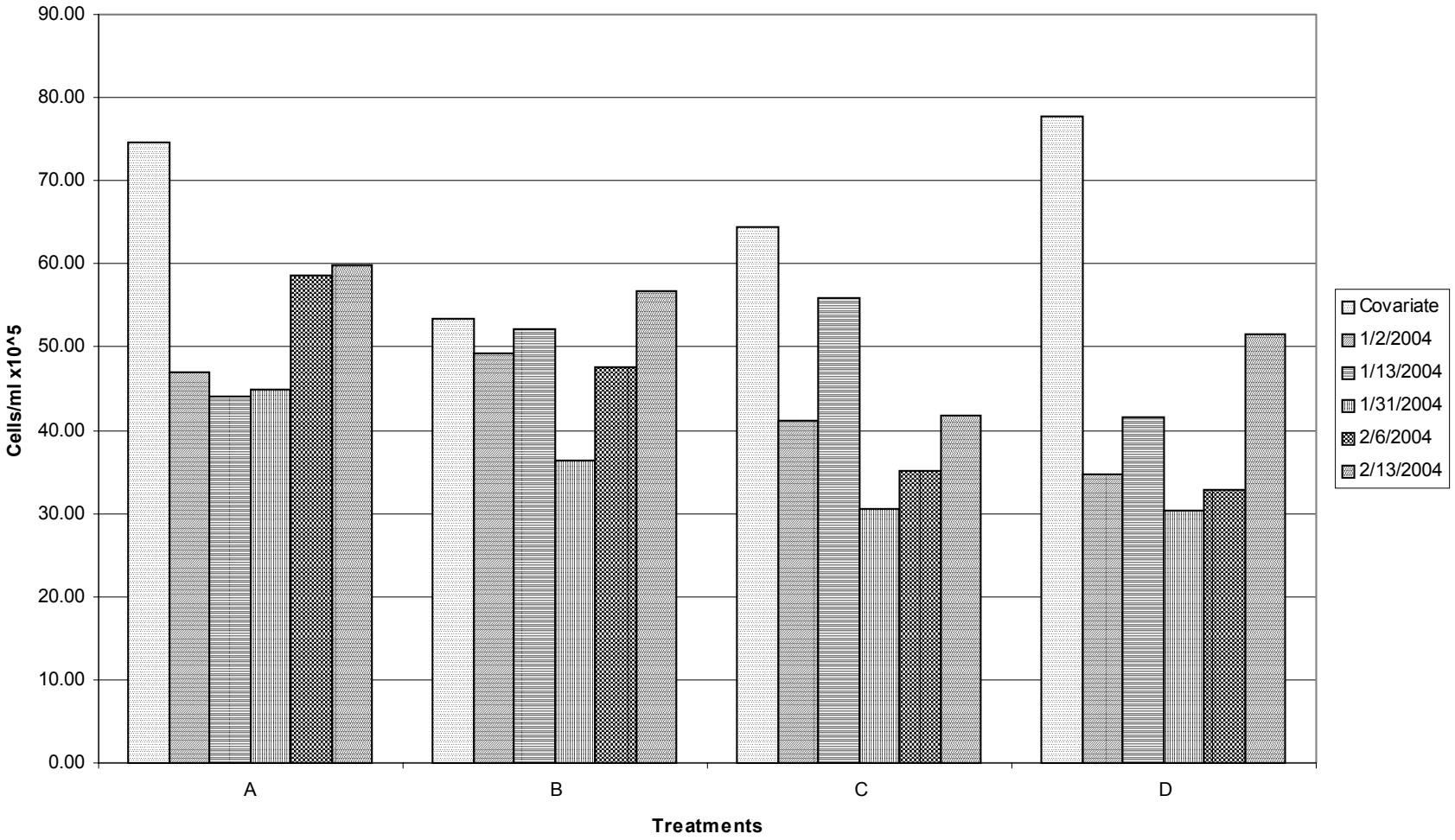

Figure 4. Effects of Lauric Acid on mean protozoal counts from experiment 2.

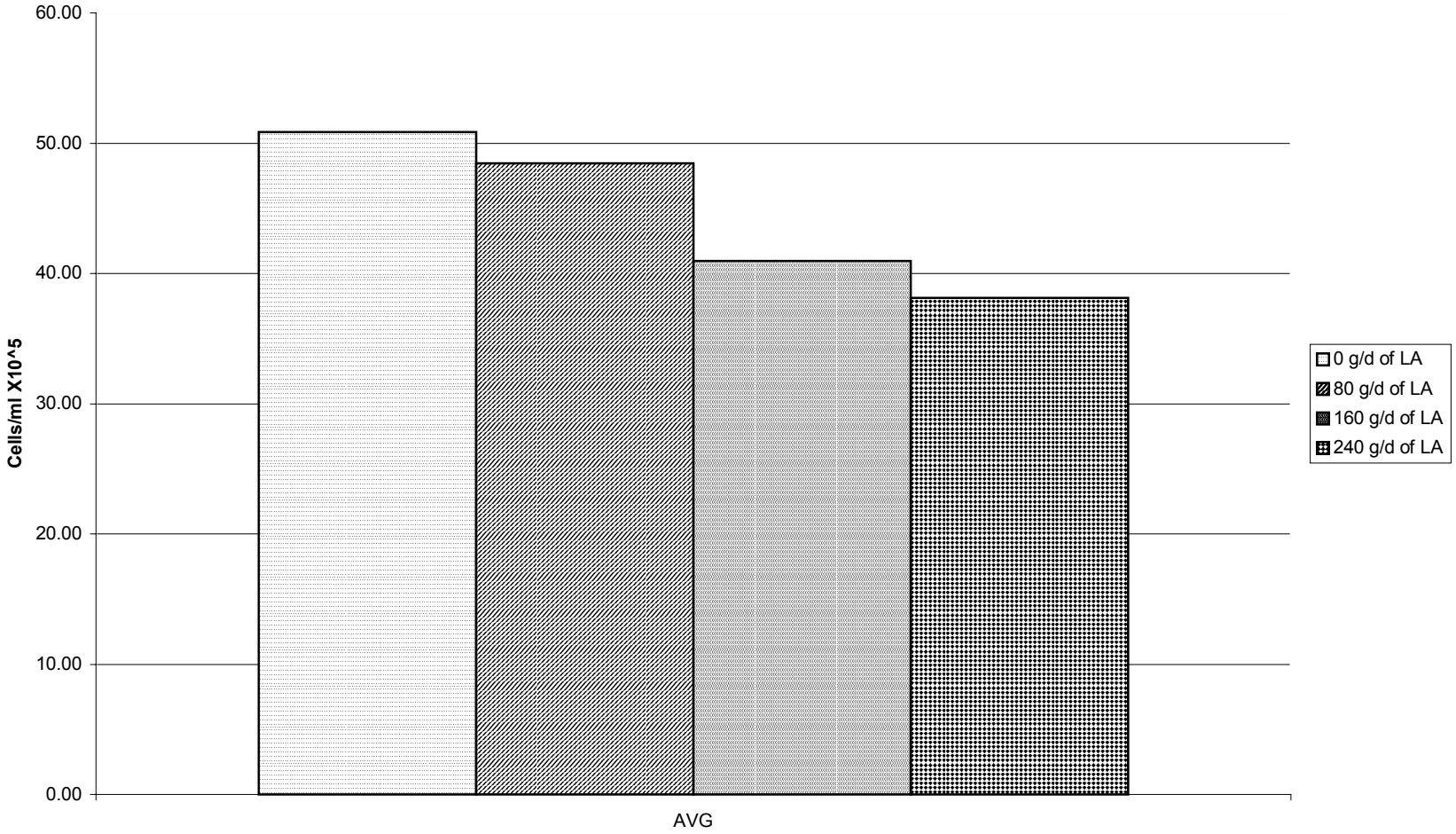

Figure 5. Milk production (last week of covariate period vs 5 last weeks of LA treatment) from experiment 2.

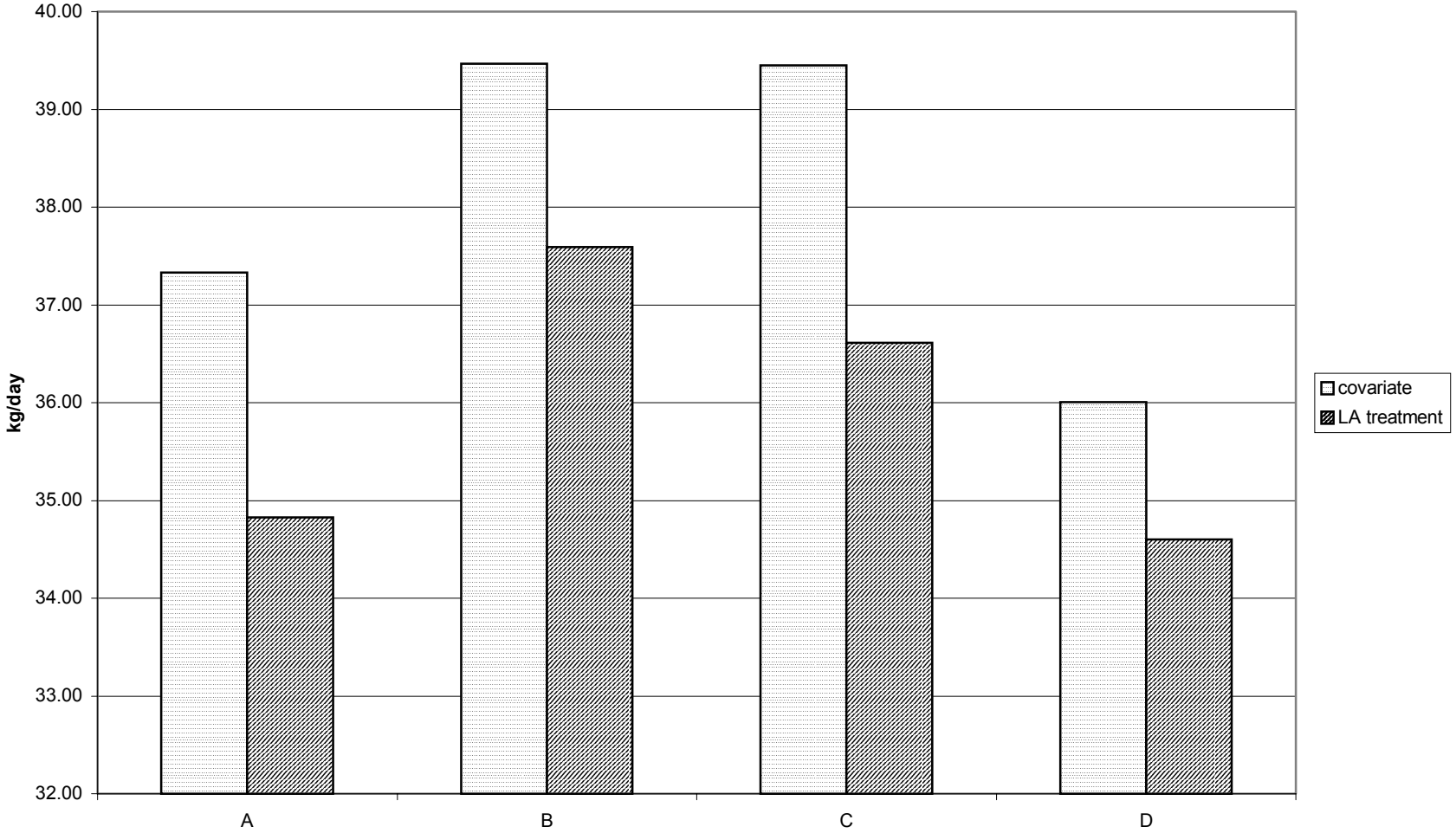

Figure 6. Milk production (all cows last week of covariate period vs 5 last weeks of LA treatment) from experiment 2.