EFFICACY OF IONOPHORES IN CATTLE DIETS FOR MITIGATION OF ENTERIC METHANE

A Thesis

Submitted to the Faculty

of

Graduate Studies

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by

Hongtao Guan

In Partial Fulfillment of the

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FACULTY OF GRADUATE STUDIES
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"Efficacy of Ionophores in Cattle Diets for Mitigation of Enteric Methane"

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ABSTRACT

Use of ionophores in cattle diets has been proposed as a mitigation strategy for enteric methane (CH_4) emissions. The short and long term effects of feeding a single ionophore (monensin) or rotation of two ionophores (monensin and lasalocid) on enteric CH_4 emissions of beef cattle were evaluated in the present study. Thirty-six Angus yearling steers (328 ± 24.9 kg) were used over the course of the 16-week feeding trial. They were randomly assigned to six dietary treatments, each with six steers. The six diets were high-forage without ionophore supplementation, high-forage with monensin supplementation, high-forage with a two-week rotation of monensin and lasalocid supplementation, high-concentrate without ionophore supplementation, high-concentrate with monensin supplementation, and high-concentrate with a two-week rotation of monensin and lasalocid supplementation, respectively. Enteric methane emissions, as measured using the SF_6 tracer gas technique, ranged from 54.7 to 369.3 L d^{-1} over the course of the study. Methane energy lost (% GEI) ranged from 1.1 to 9.2 % on the high-forage diets and from 0.9 to 8.4 % on the high-concentrate diets. Supplementing ionophores resulted in a 30% and 27% decrease ($P<0.05$) in methane emissions, expressed as L kg^{-1} DMI or % GEI, for the first two and four weeks for cattle receiving the high-concentrate and high-forage diets, respectively. No benefit related to either extent of decline in methane emissions or length of time was observed with the rotation of ionophores, compared with monensin supplementation. Total volatile fatty acid concentration of rumen fluid was not changed ($P>0.05$) as a result of ionophore addition to the diets, however, the ratio of acetate and propionate, and ammonia-nitrogen concentration in the rumen fluid were decreased ($P<0.001$) from the time ionophores

were introduced to the time they were removed from the diets. Both monensin, and rotation of monensin and lasalocid decreased (P<0.001) total ciliate protozoal populations by 82.5% and 76.8% in the first two and four weeks they were supplemented in the high concentrate and high forage diets, respectively. Original total ciliate protozoal populations were restored by the sixth week of supplementation when cattle were fed the high forage diet, and by fourth week of supplementation when cattle were fed the high concentrate diet. No significant change was observed thereafter. These data suggest that the overall effect on rumen fermentation is probably due to shifts in the microbial population towards ionophore-resistant organisms. The reductions in methane production due to ionophore supplementation were more closely related to reductions in the rumen protozoa population. Also, the data obtained in the present study suggest that ciliate protozoal populations can adapt to the ionophores presented in either high forage or high concentrate cattle diets. Furthermore, it appears that rotation of monensin and lasalocid did not avoid ciliate protozoal adaptation to ionophores.

FORWARD

This thesis was written in a manuscript format. The research conducted for the purpose of this M.Sc. program was written as the manuscript - efficacy of ionophores in cattle diets for mitigation of enteric methane. Part of manuscript was presented at the first national conference hosted by BIOCAP Canada Foundation on February 2nd and 3rd, 2005 in Ottawa (http://www.biocap.ca/images/pdfs/conferencePosters).

ABBREVIATIONS

ADF	Acid detergent fibre
ADG	Average daily gain
BW	Body weight
CH_4	Methane
CO_2	Carbon dioxide
CO_2 Equiv.	Carbon dioxide equivalents
CP	Crude protein
DM	Dry matter
DMI	Dry matter intake
DOM	Digestible organic matter
FCR	Feed conversion rate
GE	Gross energy
GEI	Gross energy intake
GHG	Greenhouse gas
GWP	Global warming potential
H_2	Hydrogen
IVDMD	In vitro dry matter digestibility
IVOMD	In vitro organic matter digestibility
ME	Metabolizable energy
Mt	Megatonnes
NDF	Neutral detergent fibre

NH$_3$-N	Ammonia nitrogen
SF$_6$	Sulphur hexafluoride
VFA	Volatile fatty acids

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1. GENERAL INTRODUCTION

Methane is the product of anaerobic fermentation of organic matter and is an important greenhouse gas, having 23 times the global warming potential of carbon dioxide (Intergovernmental Panel on Climate Change (IPCC) 2001). Livestock account for approximately 15% of global methane emissions, much of which arises from ruminant animals. In Canada, cattle account for 97% of total livestock enteric methane emission with 72% associated with beef cattle production and 25% related to dairy production (Janzen et al. 1999).

Methane produced during ruminal fermentation is not only a significant source of greenhouse gas, but also represents a substantial energy loss to the ruminants (Johnson and Johnson 1995). Therefore, reducing methane production by ruminants would have significant economical and environmental benefits.

There are a variety of factors influencing ruminal methane production, such as diet quality (Boadi et al. 2004b), level of feed intake (Johnson and Johnson 1995), feeding frequency (Sutton et al. 1986), and supplementing ionophores (Mbanzamihigo et al. 1996) or lipids in animal diets (Machmuller and Kreuzer 1999). In addition, methane production by ruminants can be affected by ambient temperature (Kennedy and Milligan 1978), and different species and breeds (Lal et al. 1987).

Considerable research efforts have been devoted to develop the strategies to mitigate methane production. Some strategies, such as biotechnological strategies, are impractical at present time. However, since ionophores have beneficial effects on feed efficiency and productivity, and are used extensively in cattle industry, the use of

ionophores as a strategy for decreasing methane emission from ruminants would be economically feasible.

Ionophores are highly lipophilic substances, which are able to shield and delocalize the charge of ions and facilitate their movement across membranes (Mathison et al. 1998). Monensin and lasalocid are two ionophores which are commonly used to manipulate rumen fermentation and, thereby, improve the efficiency of feed utilization (Russell and Strobel 1989). They have been shown to decrease methane production, increase the ratio of propionic to acetic acid production, and decrease ammonia nitrogen in the rumen (Rumpler et al. 1986; Russell and Strobel 1989; Sauer et al. 1998). However, some long-term in vivo trials have shown that depression of methane production by monensin or lasalocid did not persist, while the altered rumen VFA patterns persisted as long as ionophores were given (Rumpler et al., 1986; Carmean and Johnson 1990). This is in contradiction with the accepted stoichiometry of rumen fermentation: an increase in propionic acid production should be related to a decrease in methanogenesis (Wolin 1960).

It has been hypothesized that ionophores do not affect methanogens directly, but rather act on protozoal populations which act as a symbiotic host for methanogens. Protozoa in the rumen are indirectly associated with a high proportion of H_2 production, and are closely associated with methanogens by providing a habitat for up to 20% of rumen methanogens (Newbold et al. 1995). Defaunation has been shown to reduce ruminal CH_4 production by about 20 to 50% (Whitelaw et al. 1984). Wallace et al. (1981) reported that ruminal protozoa were inhibited by monensin in vitro, but not all researchers have observed a reduction in total ruminal protozoal numbers in vivo (Leng

et al. 1984). In addition, the number of studies investigating the effect of lasalocid on ruminal ciliate protozoa is limited. Dennis and Nagaraja (1986) observed that lasalocid decreased the total protozoal numbers both in vitro and in vivo.

Although there has been evidence of an adaptation response of microbes to ionophores fed to cattle (Saa et al. 1993; Mbanzamihigo et al. 1996), the mechanisms of ionophore resistance have not been well defined. Recent research indicates that extracellular polysaccarides play a key role in the ionophore resistance of ruminal bacteria (Rychlik and Russell 2002). While cross-resistance to ionophores is common, Chen and Wolin (1979) reported that some Bacteroides strains (methanogenic and rumen saccharolytic) were resistant to monensin but not to lasalocid and vice versa. However, there is limited information regarding ionophore combinations, synergisms and adaptations.

It was hypothesized that:

1) According to the stoichiometry of rumen fermentation: an increase in propionate production should be related to a decrease in methane production.

2) A high grain or concentrate diet could induce adaptation of rumen fermentation to ionophores.

3) Combinations of ionophores could prevent microbial adaptation or have more persistent effects on rumen fermentation.

The objectives of the present study were:

1) To evaluate the impact of the administration of ionophores on enteric CH_4 emission.

2) To discern the duration of ionophore-mediated suppression of CH_4 production and alterations in rumen fermentation.

3) To investigate the effect of the rotation of ionophores (monensin and lasalocid) on CH_4 emission and rumen fermentation.

4) To assess the effects of ionophores on concentrations of ruminal ciliate protozoa in cattle fed high-forage or high-concentrate diets.

Table. 2.1. Methane emission rates from agricultural sources[b]

Sources	CH$_4$ emission rates (Mt CO$_2$ equiv. y^{-1})[c]
Enteric fermentation	1,680
Animal wastes	525
Paddy rice production	1,260-2,100
Biomass burning	840
Total	4,305-5,145

[b]Data derived from Moss et al. (2000). Estimates based on IPCC Tier I equations, assuming CH$_4$ has a GWP of 23.

[c]Mt CO$_2$ equiv = million tonne CO$_2$ equivalents.

(parts per billion by volume) (IPCC 2001). An estimated 1,764 million tonnes CO_2 equivalent of methane per year is in excess, considering all sources and sinks of methane (Moss et al. 2000). Thus, reducing atmospheric methane concentrations would require reductions in methane emissions or increased sinks for methane.

Carbon dioxide equivalents provide a universal standard of measurement against which the impacts of releasing different greenhouse gases can be evaluated (IPCC 2001). Every greenhouse gas has a global warming potential (GWP), a measurement of the impact that particular gas has on "radiative forcing;" that is, the additional heat which is retained in the earth's ecosystem through the addition of this gas to the atmosphere. The GWP of a given gas describes its effect on climate change relative to a similar amount of carbon dioxide and is divided into a three-part "time horizon" of twenty, one hundred, and five hundred years (IPCC 2001). Global warming potentials for the greenhouse gases regulated under the Kyoto Protocol under a 100-year timeframe are as follows:

Carbon dioxide has a GWP of 1

Methane has a GWP of 23

Nitrous oxide has a GWP of 296

Halocarbons (HFC) have a GWP of 120 to 12,000

Sulphur hexafluoride has a GWP of 22,200.

Since methane has a warming potential that is 23 fold greater than carbon dioxide under a 100-year timeframe and a short atmospheric life of 8.4-12 years (IPCC, 2001), any reduction in atmospheric methane concentration will have a relatively immediate impact on global warming.

2.2. Livestock Methane Production

2.2.1. Global Livestock Methane Production Estimates

Methane gas production is a natural by-product of feed fermentation in the gastrointestinal tract of the livestock. Animal production accounts for about 20.4 % of total atmospheric CH_4 emissions, and is considered to be the largest source of methane from human-related activities (Houghton et al. 1992). Gibbs and Johnson (1994) estimated livestock CH_4 production to be 1,663.2 million tonnes CO_2 equivalent per year (Table 2.2). Methane production by the world's population of ruminants was 1,617 million tonnes CO_2 equivalents annually, which constitutes about 97 % of CH_4 produced by domestic animals (Johnson and Ward 1996). Cattle produce about 75% of total livestock CH_4 emissions, while buffalo, sheep, and goats produce 9-10 %, 9 % and 3.8-3.9 % of total animal emissions, respectively (Johnson and Ward 1996). Therefore, ruminant production has been a main target for reducing global CH_4 emissions.

Methane production represents a substantial energy loss to ruminants of about 2-12% of gross energy intake (Johnson and Johnson 1995). Thus, reducing CH_4 emissions from the ruminant will not only reduce the contribution to the atmospheric CH_4 concentration, but also will improve production efficiency.

2.2.2. Canadian Livestock Methane Production Estimates

Canadian livestock produce about 1% of the global methane production attributable to livestock (McAllister et al. 1996). Methane generated by enteric fermentation contributes approximately 18 million tonnes CO_2 equivalents of total global greenhouse gas emissions in 1996, and has increased by 14% since 1990 due to an increase in cattle production (Neitzert et al. 1999). In 2001, methane emissions from

Table 2.2. Estimates of global enteric methane emissions by livestock[b]

Species	CH_4 (Mt CO_2 equiv. y^{-1})[c]	% of total emissions
Cattle	1220.1	73.3
Buffalo	161.7	9.7
Sheep	149.1	8.9
Goats	58.8	3.5
Camel	18.9	1.1
Pigs	23.1	1.4
Equine	35.7	2.1
Total	1667.4	

[b]Data derived from Gibbs and Johnson (1994). Estimates based on the estimates of CH_4 production from individual animal and animal populations in different parts of world.
[c]Mt CO_2 equiv = million tonne CO_2 equivalents.

enteric fermentation and manure management were 31.3 and 16.8 %, respectively, of the total CO_2 equivalents emissions from Canadian agriculture (Environment Canada 2003). Methane emissions from beef and dairy production contribute approximately 97% of total enteric fermentation emissions for Canada (Janzen et al. 1999).

The above estimations of methane emissions have been based on IPCC Tier I equations, which multiply the population of each animal category by a single-value emission factor associated with the specific animal category (IPCC 1997). However, the accuracy of these estimates is questionable because animal type, physiological status, age, gender, weight, rate of gain, level of activity, and environmental conditions can affect the amount of methane generated by livestock production. According to IPCC (2000), using IPCC Tier II methodology, which accounts for weight, age, gender, and feeding system, can improve emission estimates and reduce uncertainties. Boadi et al. (2004a) used IPCC Tier II equations to estimate that enteric methane emissions by Canadian beef cattle and dairy cattle are approximately 16 and 3.6 million tonnes CO_2 equivalent in 2001, respectively (Table. 2.3).

2.3. Rumen Methanogens

Methanogens are a diverse group of organisms that can live in a wide range of environments such as sanitary landfills, acidic peat bogs, waterlogged soils, salt lakes, and the digestive tract of animals (Zinder 1993). They are strict anaerobes and can be unambiguously differentiated from other organisms since they all produce methane as a major catabolic product (Woese et al. 1990). Methanogens are members of the domain Archaea, belonging to the kingdom Euryarchaeota. Nineteen genera and more than 60 species of methanogens have been described (Hales et al. 1996). The diversity of the

Table 2.3. Summary of Canadian cattle enteric CH_4 emissions (10^3 tonnes CO_2 equiv.) by province in 2001 using IPCC Tier II equations[b]

	Beef cattle	Dairy cattle
British Columbia	890.1	276.4
Alberta	7,483.2	299.3
Saskatchewan	3,155.0	99.2
Manitoba	1,603.2	147.9
Ontario	1,753.1	1,305.8
Quebec	925.9	1,294.1
Newfoundland	4.0	14.9
New Brunswick	76.2	68.0
Nova Scotia	86.8	78.7
Prince Edward Island	54.7	49.3
Total	16,032.2	3,633.6

[b]Data derived from Boadi et al. (2004a)

methanogens is demonstrated by the large differences in cell envelope structure. The three known types of cell walls are composed of pseudomurein, protein, and heteropolysaccharide, respectively (König 1988). It is recognized that methanogens lack murein, which is typical of almost all bacteria (König 1988). In addition, methanogens possess three coenzymes, which have not been found in other microorganisms. The three coenzymes are: coenzyme F_{420}, involved in two-electron transfer reactions; coenzyme M, serving as the terminal carbon carrier in methanogenesis; and factor B, a low molecular weight, oxygen-sensitive, heat-stable coenzyme participating in the enzymatic formation of methane (Baker 1999). Analyses of the nucleotide sequence of the 16S ribosomal RNA indicate that methanogens diverge from eubacteria near the time of the origin of life during evolution (Olsen and Woese 1997).

Since methanogens are nutritionally fastidious anaerobes, media for them have to maintain a redox potential below –300 mV (Stewart and Bryant 1988). Although most methanogens have a pH optima near neutral, there are some methanogens that live in extreme pH (3 - 9.2) environments (Jones et al. 1987). Methanogenesis is greatly limited when the surrounding temperature is below 15 ^{0}C, but can still occur readily at temperatures near 100 ^{0}C. Methanogens are found in both of these environments. *Methanoculleus submarinus* is found at temperatures near 15 ^{0}C, while *Methanopyrus kandleri* is found at temperatures in the other end of the spectrum. Thermophilic methanogens, such as *Methanococcus jannaschii* and *Methanothermobacter thermoautotrophicum*, grow at temperature of 85 ^{0}C (Jones et al. 1987).

Five species of methanogens, belonging to *Methanobrevibacter* and *Methanosarcina* genera, were reported to have been isolated in the rumen so far

(McAllister et al.1996). They are *Methanobrevibacter ruminantium*, *Methanosarcina barkeri*, *Methanosarcina mazei*, *Methanobacterium formicicum* and *Methanomicrobium mobile*. However, only *Methanobrevibacter ruminantium* and *Methanosarcina barkeri* have been found in the rumen at a population level greater than 10^6 ml^{-1} (Lovley et al. 1984). In recent years, phylogenetic analysis of Archaeal 16S rRNA genes (rDNA) amplified from total DNA extracted from rumen fluid showed that most of the organisms represented methanogens not previously recovered from the rumen or differed from the cultivated species (Whitford et al. 2001). Furthermore, new species are frequently found from other anaerobic habits (Jones et al. 1987). Thus, it is almost certain that there are more rumen methanogens not yet identified, and that more species will be identified as 16S rRNA analysis progresses.

Some methanogens are frequently found in association with ciliate protozoa in the rumen (Finlay et al. 1994; Tokura et al. 1999). A recent study using 16S rRNA probes directed against different families of methanogens indicated that methanogens associated with rumen ciliate protozoa were highly related to *M. smithii* and. *M. ruminantium* (Whitford et al. 2001). They either live on the exterior surface of rumen ciliate protozoa or as endosymbionts within the cilates (Moss et al. 2000). However, the association of methanogens with rumen ciliate protozoa seems to be dependent on the time of feeding the animal. Tokura et al. (1997) observed that the number of methanogens attached to protozoa was greatest soon after feeding, which was 10 to 100 times greater than pre-feeding attachment.

It has been recognized that the symbiotic relationship of methanogens and rumen ciliate protozoa could contribute 30 to 45% of methane production from the rumen

(Tokura et al. 1999). Thereby, this association of methanogens with protozoa could be used to decrease the methane production through removing protozoa or decreasing the protozoal populations in the rumen microbial ecosystem. Although methanogens are only directly involved in the very terminal stages of fermentation, they are very important because they are capable of effectively preventing H_2 accumulation in the rumen by utilizing electrons in the form of H_2 to reduce CO_2 to CH_4, thereby enhancing the energetic efficiency and extent of organic matter digestion by other ruminal microorganism. Thus, in their absence, organic matter could not be degraded as effectively in the gut (McAllister et al. 1996).

2.4. Rumen Protozoa

Protozoa, considered as the simplest form of animal life, are the most abundant animals in the world in terms of numbers and biomass. Their principal importance is as consumers of bacteria. Bacteria play a vital role in maintaining the earth as a suitable place for inhabitation by other forms of life and protozoa play a vital role in controlling their numbers and biomass. Protozoa are also important as parasites and symbiont multicellular animals. Over 60,000 protozoal species, belonging to four major groups (flagellates, amoebae, sporazoans, and ciliates) have been described (Dehority 2003).

Rumen protozoa have evolved into a highly specialized group, which only survive in the rumen or a closely related habitat. They are anaerobic, can ferment plant material for energy, and can grow at rumen temperatures in the presence of billions of accompanying bacteria (Hungate 1966). Rumen protozoa vary considerably in size, ranging from approximately 15 to 250 μm in length and 10 to 200 μm in width for the different species (Dehority 2003). It has been observed that a majority of rumen protozoa

are ciliate species with about 90 % *Entodinium*, while flagellates occur in relatively low numbers in adult ruminants and are quite small in size as compared to the ciliates (Clark 1977; Corliss 1994). All rumen ciliate protozoa are classified in the phylum Ciliophora. However, numerous changes in the system of classification below the phylum level have resulted from detailed ultrastructural studies, and the application of molecular techniques analyses (Corliss 1994). Based on the most recent revision, rumen protozoa are classified to six families, including twenty-four species (Dehority 2003). The six families are *Isotrichidae, Paraisotrichiae, Blepharocorythinae, Buetschliidae, Ophryoscolecidae,* and *Cycloposthiidae.*

Two factors seem to be of prime importance in establishment of a ciliate protozoal population in the rumen. First, the animal is exposed to an adult or faunated animal. Second, the environmental conditions within the rumen are satisfactory for survival of the protozoa (Dehority and Orphin 1988). As an overall estimate, a protozoan concentration of 1×10^6 per ml of rumen contents appears to be "normal" for both cattle and sheep (Dehority 2003). However, several factors may influence the concentration and composition of the protozoal fauna in the rumen. These include composition of diet, pH, turnover rate, frequency of feeding, and feed level, addition of fat and supplement of ionophores in the animal diets. Franzolin and Dehority (1996) reported that feeding high-concentrate diets resulted in a reduction of rumen pH below 6.0 with a marked decrease in protozoal concentrations, while primarily *Entodinium* species were present. Earlier observations with ruminants fed high-concentrate diets suggested that protozoa were practically eliminated, especially in ad libitum conditions, because of the acidic conditions in the rumen (Eadie et al. 1970). However, recent studies have shown that

protozoa may be present in significant numbers in feedlot cattle fed a variety of grains (Towne et al. 1990). It seems that rations containing between 40 and 60% concentrate will support maximal protozoan numbers with a diverse fauna containing species in most of the genera (Dehority and Orpin 1988). The discrepancies between the different studies suggest that in addition to pH, other factors, such as feed level, feeding regime and diurnal variation, are involved in influencing the numbers of protozoa in the rumen.

Ruminal ciliate protozoa play diverse and important roles in ruminal metabolism of nutritients. On the other hand, since the flagellates seem to occur in relatively low numbers and only utilize soluble substrates, their contribution to the overall rumen fermentation could be minimal (Clark 1977). The best-studied role of protozoa in the rumen is their negative role in rumen nitrogen metabolism. Engulfment and digestion of bacteria by ciliate protozoa in the rumen is responsible for the majority (over 80%) of the microbial recycling in the rumen, while ciliate protozoa themselves tend to sequester within the rumen and do not make a major contribution to the flow of nitrogen from the rumen (Ushida et al. 1986). Furthermore, in vivo trials have confirmed that duodenal flow of both undegraded dietary protein plus bacterial protein generally is increased by defaunation (Jouany 1996). In addition, protozoa may moderate rumen fermentation in high-grain fed steers through random predation of the rumen bacteria. Nagaraja et al. (1992) observed that high-grain fed, protozoa-free steers had lower ruminal pH values than faunated cattle (pH 5.97 vs pH 6.45), about four-fold higher bacterial concentrations and higher VFA concentrations. Chaudhary et al. (1995) measured digestibility of forage structural carbohydrates in water buffalo fed wheat straw plus a concentrate mixture. The defaunation was accomplished by introducing Manoxol (BOH, London, UK) into the

rumen at a rate of 10g Manoxol per 100 kg body weight for two successive days. The authors concluded that defaunation decreases the digestibility of structural carbohydrates, both in the rumen and total gastrointestinal tract.

The importance of methanogens associated with ciliate protozoa was investigated by Newbold et al. (1995). They estimated that about 9 to 25% of the methane produced in the rumen was by endosymbiotic methanogens associated with protozoa. In addition, Ushida and Jouany (1996) observed that the daily production of methanogens associated with a single protozoal cell ranged from a trace to 2 nmol.

2.5. Substrates and Energetics of Methanogenesis

Methanogens obtain their energy for growth from the conversion of a limited number of substrates to CH_4. The major substrates are H_2, CO_2, formate, and acetate. In addition, some other compounds such as methanol, methylamines, and dimethyl sulfide are substrates for some methanogens (Jones 1991; Table 2.4). From Table 2.4, it can be seen that the change in free-energy ($\triangle G$) of the H_2-utilizing reaction is the most negative (-135 kJ mol^{-1} of methane). It indicates that methanogens have very high affinity for H_2, since the reaction is the most thermodynamically favourable.

Methanogens primarily use H_2, CO_2 and formate as substrates for methanogenesis in the rumen (Jones 1991). Only two species (*Methanosarcina* and *Methanosaeta*) are able to catabolize acetate to CH_4 in the rumen (Jones 1991). However, since there is only a small change (-31 kJ mol^{-1} of methane) in free-energy ($\triangle G$), this pathway represents an energetically unfavourable pathway for methanogens. Thus, if better growth substrates are available, methanogens will not utilize acetate for

biosynthesis and produce CH_4 through a more energetically favourable pathway (Jones et al. 1987). This may explain why all methanogens prefer utilizing H_2 to reduce CH_4, the most energy-yielding pathway.

2.6. Biochemistry of Methanogenesis

Methanogens convert CO_2 to CH_4 through four reductive intermediates: fromyl, methenyl, methylenyl and methyl. Six coenzymes identified so far are involved in the reduction of CO_2 to CH_4 (DiMarco et al. 1990). These coenzymes include methanofuran (MFR); tetrahydromethanoopterin (H_4MPT); cofactor F_{420}; cofactor F_{430}; coenzyme M (CoM) and 7 mercaptoheptanoylthreonine (HS-HTP).

The pathway for methane production from CO_2 (Figure 2.1) has been described by Rouviere and Wolfe (1988) as follows: Formyl-MFR, the first stable intermediate of methanogenesis, is formed through fixing CO_2 with MFR (reaction 1). The formyl group is then transferred to H_4MPT (reaction 2), which serves as the carrier for the fromyl, methenyl, methylenyl and methyl groups. The formyl group is next converted to a methenyl group by the enzyme, 5,10-methenyl-H_4MPT cyclohydrolase (reaction 3). The reduced deazaflavin, coenzyme F420, participates in reduction of methenyl-H_4MPT to methylenyl-H_4MPT (reaction 4) and of methylenyl- H_4MPT to methyl-H_4MPT (reaction 5). Prior to reduction of the methyl group to CH_4, the methyl group of methyl-H4MPT is transferred to CoM (reaction 6). Methyl-CoM is then reduced to methane by methyl-coenzyme methyl reduatase, a complex system composed of proteins and a number of cofactors such as F430, ATP, HS-HTP, FAD (reaction 7). This reaction terminates the cycle and is linked to the activation of CO_2 to form formyl-MFR.

Methanogenesis using acetate as a substrate, as described by Jones (1991), also proceeds through the methlreductase system. First, acetate is activated to acetyl coenzyme A. Acetyl coenzyme A is then metabolized to an enzyme-bound carbon monoxide and a methyl group. The methyl group is next transferred to HS-CoM to form methyl-CoM, which is accomplished by H_4MPT. The oxidation of enzyme-bound carbon monoxide donates electrons for the reductive cleavage of methyl-CoM to CH_4.

Jones (1991) has also described conversion of methanol and methylamines via two pathways. One is direct reduction of methyl group (via H_2) and another is by a disproportion reaction. In both cases, reducing equivalents for subsequent reduction of the methyl group to CH_4 are derived from oxidation of some of the methanol and methylamines by the methylreductase system.

2.7. Fermentation in the Rumen

Since the rumen is an anaerobic ecosystem, ruminal microorganisms hydrolyze proteins, starch and plant cell wall polymers into amino acids and sugars to obtain energy and nutrients required for their growth. Ruminal gram-positive bacteria are involved in fermentation processes producing acetate, butyrate, lactate and ammonia, while ruminal gram-negative bacteria are engaged in fermentation pathways associated with the production of propionate and succinate (Russell 1996). Some of the end of products of ruminal fermentation, such as volatile fatty acids and microbial protein, are major sources of nutrients (i.e. energy and nitrogen) for the ruminants. On the other hand, other

Table 2.4. Reactions and standard changes in free energies for methanogenesis[z]

Reactions	ΔG (kJ mol^{-1} of CH_4)
Hydrogenotrophic reactions	
$\quad 4H_2 + CO_2 \rightarrow CH_4 + 2H_2O$	-135.6
$\quad 4\ Formate \rightarrow CH_4 + 3CO_2 + 2H_2O$	-130.1
$\quad 4(2\text{-propanol}) + CO_2 \rightarrow CH_4 + 4acetone + 2H_2O$	-36.5
Aceticlastic reaction	
$\quad Acetate \rightarrow CH_4 + CO_2$	-31.0
Disproportionation reactions	
$\quad 4\ Methanol \rightarrow 3CH_4 + CO_2 + 2H_2O$	-104.9
$\quad 4\ Methylamine + 3H_2O \rightarrow 3CH_4 + CO_2 + 4NH_4^+$	-75.0
$\quad 2\ Dimethyl\ sulfide + 2H_2O \rightarrow 3CH_4 + CO_2 + H_2S$	-73.8

[z]Data derived from Jones (1991)
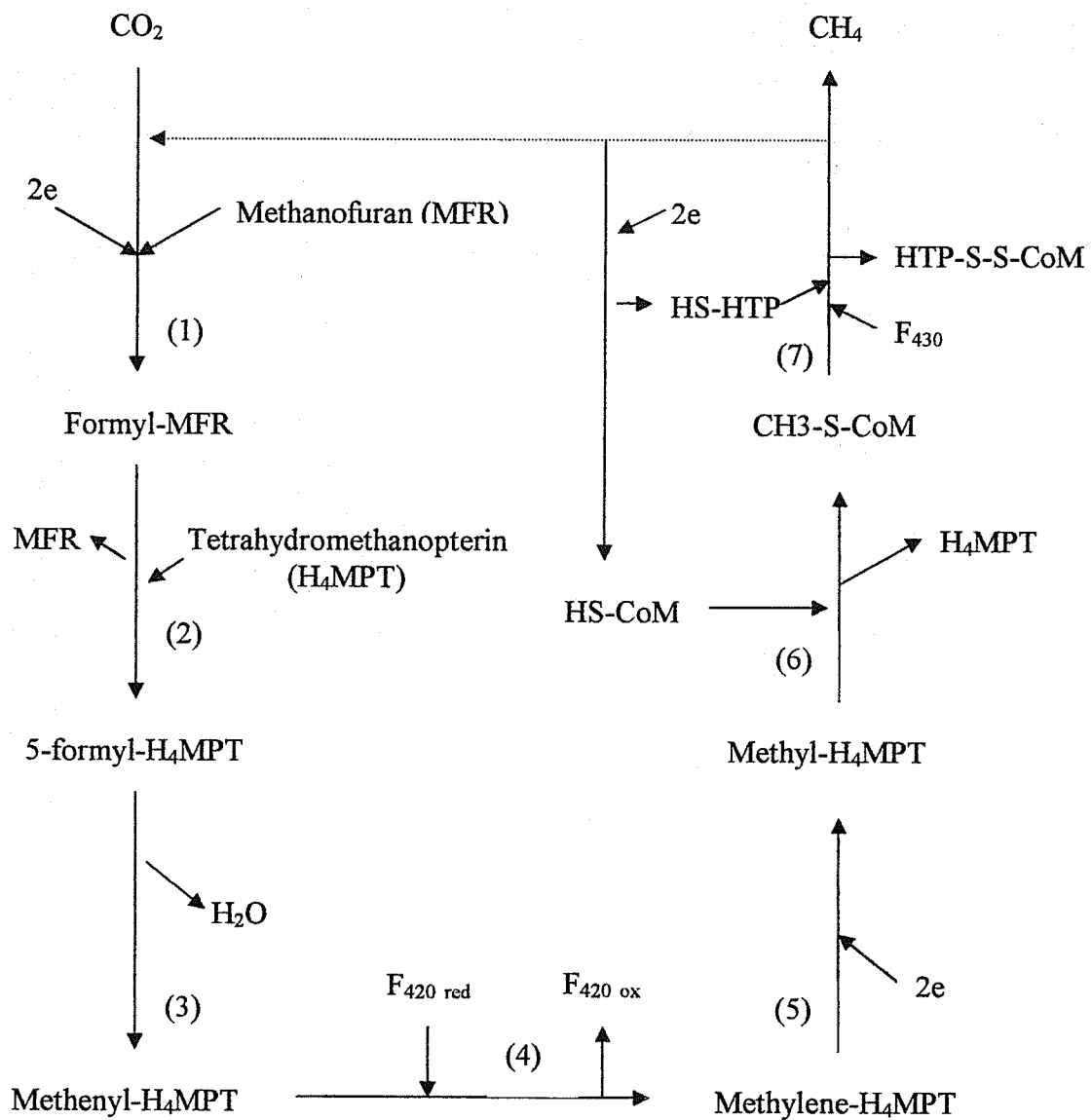
Figure 2.1. Pathway for CH₄ production from CO₂ (Modified from Rouviere and Wolfe 1988)

fermentation end products, such as methane and ammonia, represent a loss of feed energy and protein from the ruminants into the environment (Owens and Goetsch, 1988). Methane production in the rumen involves the integrated activities of different microbial species, with the final step carried out by methanogenic bacteria (McAllister et al. 1996). Primary digestive microorganisms (i.e. bacteria, protozoa and fungi) hydrolyze proteins, starch and plant cell wall polymers into amino acids and sugars. These simple products are then fermented to VFA, H_2, and CO_2 by both primary and secondary digestive microorganisms. The major producers of H_2 are the ruminal microorganisms which produce acetic acid in the fermentation pathway (Hegarty and Gerdes 1998).

$$C_6H_{12}O_6 + 2H_2O \rightarrow 2C_2H_4O_2 \text{ (acetate)} + 2CO_2 + 8H$$

$$C_6H_{12}O_6 + 4 H \rightarrow 2C_3H_6 O_2 \text{ (propionate)} + 2H_2O$$

$$C_6H_{12}O_6 \rightarrow C_4H_8 O_2 \text{ (butyrate)} + 2CO_2 + 4H$$

$$CO_2 + 8H \rightarrow CH_4 + 2H_2O$$
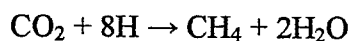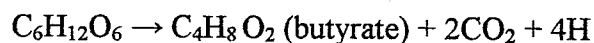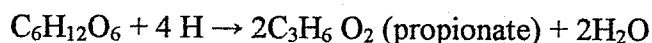
Although H_2 is one of the major end products of fermentation by ruminal bacteria, protozoa and fungi, it does not accumulate in the rumen. It is used by other bacteria, mainly the methanogens which reduce CO_2 with H_2 through methanogenesis. Moss et al. (2000) established that CH_4 production can be calculated from the stoichiometry of the main VFA formed during fermentation, i.e., acetate (C_2), propionate (C_3) and butyrate (C_4) as follows: $CH_4 = 0.45\ C_2 - 0.275\ C_3 + 0.40\ C_4$, assuming that the amount of H_2 produced is equal to H_2 used, and the recovery rate of hydrogen is 90%. This equation indicates that the molar percentage of VFA influences the production of

CH_4. Acetate and butyrate promote CH_4 production, while propionate formation serves as a competitive pathway for H_2 use in the rumen.

2.8. Factors Influencing Methane Production

2.8.1. Dietary Considerations

2.8.1.1. Diet Quality

Methane production can be affected by dietary factors such as the digestibility of the feed and the type of carbohydrates (structural vs. non-structural carbohydrates). It is well known that structural carbohydrates (i.e. cell wall fiber) are fermented within the rumen and passed out of the rumen at a slower rate than non-structural carbohydrates (i.e. sugars and starch). As a result, fermentation of structural carbohydrate yields more methane. This was clearly illustrated in trials with cattle receiving rations varying in hay to concentrate ratio from 100:0 to 20:80 (Ørskov et al. 1968). Methane production was gradually decreased with increasing amounts of concentrates, with the 20:80 ration generating half the methane production of the 100% hay diet. Johnson and Johnson (1995) reported that the level of methane losses were 6-7% and 2-3% of energy intake when cattle were fed high forage at maintenance and when cattle were fed high grain concetrates ad libtium, respectively.

Since proportions of the individual VFAs produced in the rumen are affected by the composition of the diet, mainly by the nature and rate of fermentation of carbohydrate, these characteristics have large effects on methane production. Diets rich in starch which favor propionate production decrease the methane production (Johnson and Johnson 1995). The reverse is true in high forage diets at high intake levels, which favor

a higher acetic:propionic acid ratio resulting in higher amount of methane (Hegarty and Gerdes 1998). High grain diets also lower ruminal pH, which inhibits the growth of methanogens and protozoa (Hegarty 1999).

Methane production (% of GEI) in cattle increase with maturity of forage fed (Robertson and Waghorn 2002), and is higher when forage is dried than when it is ensiled (Sundstol 1981). Grinding or pelleting of forages to improve the utilization by ruminants has been shown to decrease CH_4 losses per unit of feed intake by 20-40% when fed at high intakes (Johnson et al. 1996). Chemical treatment of cereal straws with NaOH reduces the methane production, expressed as per unit digestible organic matter (Moss et al. 1994). These studies indicate that properties of forage can also influence methane production. This can be attributed to decrease in rate of digestion within the rumen or passage rate from the rumen.

2.8.1.2. Level of Intake and Feeding Frequency

In general, methane production (% of GEI) will decrease as daily intake increases (McAllister et al. 1996). Johnson and Johnson (1995) reported that the percentage of dietary GE loss as methane declined by an average of 1.6% per level of intake, when the daily feed consumed by any given animal increased. In addition, Blaxer and Clapperton (1965) noted that there was a negative regression between methane production (% of GEI) and feeding level in 48 trials. This is caused mainly by the short retention time of digesta in the rumen. Consequently, the extent of microbial access to organic matter is decreased, which in turn decreased the extent and rate of ruminal dietary fermentation (Mathison et al. 1998). Also, a rapid passage rate favors propionate production, which is a competitive pathway for disposal of H_2 produced in the rumen.

Low feeding frequencies tend to increase molar proportions of propionate and decrease methane production (Sutton et al. 1986). This effect is caused mainly by lowering the population of ruminal protozoa, methanogens and fungi, since low feeding frequency increases diurnal fluctuations in ruminal pH (Shabi et al. 1999). On the other hand, more frequent feeding resulted in an increasing ratio of acetate to propionate and preventing drastic fluctuation in ruminal pH (French and Kennelly 1990). Kaufmann et al. (1980) observed that ruminal pH ranged from 5.85 to 6.65 when a given level of concentrate was fed to dairy cows twice a day, while ruminal pH ranged only from 6.15 to 6.40 when cows were fed six times a day.

2.8.1.3. Addition of Fats

Adding fats into ruminant diets has been shown to depress CH_4 production (Mathison 1997; Machmuller and Kreuzer 1999). An in vitro study by Dohme et al. (2000) showed that CH_4 production was reduced by 34, 21 and 20% due to addition of 53 g kg^{-1} DM of palm kernel oil, coconut oil, and canola oil, respectively, and the medium chain fatty acids (C8–C16) caused the greatest reduction in methane production. Machmuller and Kreuzer (1999) reported that when coconut oil was fed to sheep at dietary levels of 3.5 and 7%, daily methane production was reduced by 28 and 73%, respectively. A feedlot study by Mathison (1997) showed that with addition of 4% canola to a high (85%) concentrate diet, methane production was reduced by 33 %.

The depression in CH_4 production with addition of fats could be attributed to several mechanisms. These fatty acids can serve as electron acceptors during biohydrogenation in the rumen (Hegarty 1999). Long-chain fatty acids are non-fermentable and, therefore, may decrease the percentage of CH_4 that can be produced

(Johnson and Johnson 1995). Also, saturated fatty acids such as coconut oil have been shown to have a toxic effect on protozoa. Machmuller and Kreuzer (1999) observed that feeding 3.5 and 7% coconut oil reduced ciliate protozoa to 1.0×10^5 ml^{-1} and 0.3×10^5 ml^{-1}, respectively, compared to the controls (1.7×10^5 ml^{-1}).

2.8.1.4. Ionophore Supplementation

Ionophores such as monensin and lasalocid have been shown to inhibit methane production by mixed ruminal microorganisms in vitro (Fuller and Johnson 1981). Van Nevel and Demeyer (1996) found that monensin supplemented to cattle diets reduced methane production by 25% when averaged over 6 studies. The observed decrease in methane production has been associated with a shift in bacterial population from gram-positive to gram-negative bacterial with a concurrent shift in the fermentation from acetate to propionate (Moss et al. 2000). Thus, ionophores are not toxic to methanogens themselves, but to the bacteria that provide substrate to the methanogens.

There is evidence of an adaptation response of ruminal microorganisms to ionophores supplemented to cattle diets. Some long-term in vivo trials have shown that inhibition of methanogenesis by monensin and lasalocid did not persist. The control values were restored after two weeks (Rumpler et al. 1986; Johnson et al. 1997). According to stoichiometry of rumen fermentation (Wolin 1960), this is in contradiction with the observation that an altered pattern of volatile fatty acid production persisted in ionophore-treated animals during long-term trials (Rogers et al. 1997). However, Mbanzamihigo et al. (1996) observed that due to monensin supplementation, a decrease in methane production and altered fermentation of volatile fatty acid production persisted for 35 days. While cross-resistance to ionophores is common, Chen and Wolin (1979)

reported that some Bacteroides strains (methanogenic and rumen saccharolytic) were resistant to monensin but not to lasalocid and vice versa. In addition, Wakita et al. (1986) reported that the effect of salinomycin on methane production seems to be more persistent. Therefore, future potential lies in the use of more persistent ionophores, and combinations and synergisms of ionophores that can avoid microbial adaptation.

2.8.1.5. Chemical Supplementation

It has been shown that chlorinated methane analogues (i.e. chloroform, carbon tetra-chloride and methylene chloride) (Bauchop 1967) and related compounds such as trichloroethyl adipate (Clapperton 1977) and pyromellitic diimide (Linn et al. 1982) inhibited methnogensis by directly inhibiting methanogens. Prins et al. (1972) reported that methane production by pure strains of methanogens was inhibited by chloroform and carbon tetra-chloride. McCrabb et al. (1997) observed that a chemical complex of bromochloromethane (BCM) and cyclodextrin (CD) significantly reduced methane production without any negative effect on fiber digestion in vivo. However, microbial populations in vivo have been found to adapt to or degrade many of these compounds so that the effects of these chemicals on ruminal methanogensis were transient (Van Nevel and Demeyer 1996). In addition, there remain some concern regarding the use of these compounds, since they may have negative effects on animal health and environment.

Some compounds such as sulphate and nitrate can depress methane production as alternate electron acceptors (Itabashi 2002). These compounds divert electrons from the reduction of carbon dioxide to other acceptors. This is confirmed by the fact that reduction of nitrate and sulphate are thermodynamically favored over reduction of carbon dioxide, which provides nitrate- and sulphate-reducing microorganisms a competitive

advantage over methanogens for hydrogen (Itabashi 2002). However, these compounds cannot be recommended for use as methane inhibitors, since they may poison the animals (Nakamura et al. 1981).

It has been found that some organic acids such as fumaric acid depressed methane production by 6% in vitro (Lopez et al. 1999a). More recently, an in vivo study by Erudenbayaru et al (2001) showed that when fumaric acid was added to either high forage of high concentrate diet, a greater reduction of methane production by 20% was related to the use of high forage diet, which normally gives rise to large quantities of methane and low proportions of propionate in the rumen. Since administration of organic acids not only decreases methane production, but increases plasma glucose concentration and net energy retention, these compounds could be practically used in ruminant diets.

2.8.1.6. Biological Agents

As discussed above, the range of chemical inhibitors of methanogenesis cannot be widely applied in ruminant industries, due to the risk to the environment and animal health. Thus, it is apparent that the ruminant sector needs to develop biological tools for stable, safe, and long-term inhibition of methanogenesis. It has been shown that biological agents (i.e. phages, bacteriocins and acetogens) could be used to control ruminal methanogenesis and would be applicable for long-term rumen modification (Klieve and Hegarty 1999).

Phages are obligate pathogens that can infect and lyse bacteria and archaea, potentially depressing methanogensis (Klieve and Hegarty 1999). Studies on the host-range of phages of the common ruminal bacteria (Klieve et al. 1991) indicate that many of the phages are strain specific. Nevertheless, Newbold et al. (1996) reported that

substantial lysis of *Methanobrevibacter* MF1 in cell-free rumen fluid was caused by archaeal phage. Furthermore, filamentous and pleomorphic archaeal phages have astonishing variety, indicating that more novel phage families could be found in methanogens (Ackermann 2000). However, assessing archaeal phage potential as biocontrol agents needs considerable increase in the knowledge of the genetic diversity and viral susceptibility of archaeal methanogens and the host range of archaeal phages (Klieve and Hegarty 1999).

Bacteriocins, such as nisin, are bacteriocidal compounds that are produced by bacteria, and are generally peptide or protein in nature. Callaway et al. (1997) reported that nisin stimulated propionate production and reduced methanogenesis in vitro by 36%. It is known that nisin mainly acts on the cytoplasmic membranes of susceptible microorganisms, resulting in the dissipation of the proton motive force. Its dissipation depletes intracellular ATP and blocks amino acid uptake, which cause microorganisms to die (Montville and Chen 1998). However, whether the reduction of ruminal methanogenesis is a direct effect of nisin on methanogens or on bacteria supplying substrate to methanogens remains to be determined. There are some significant advantages for the use of bacteriocins, such as the long history of safe use, susceptibility to proteolytic digestion and possibility of genetic transfer and manipulation (Klieve and Hegarty 1999). As such, they might provide an effective alternative to the ionophores supplemented in ruminant diets.

It is well known that both methanogenesis and acetogenesis are processes utilizing hydrogen to reduce carbon dioxide. In order to divert hydrogen and carbon dioxide metabolism towards acetogenesis rather than methanogenesis, one of the

strategies is the addition of reductive acetogens to the rumen to directly compete with methanogens. Research conducted by Lopez et al. (1999b) showed that two acetogens, *Eubacterium limosum* ATCC 8486 and *Ser* 5, decreased methane production by about 5% in vitro. Although the effect of adding acetogens on methane production was minimal, in this study it supports the hypothesis that the addition of reductive acetogens might be possible to control methanogenesis. More importantly, acetogens which use hydrogen for growth are present in ruminants (Joblin 1999), so opportunities for successfully establishing acetogens in the rumen should exist.

2.8.2. Environmental Considerations

Generally, ruminal methane production would be less at low ambient temperatures than at high ambient temperatures based on the physiology of ruminant animals. This could be attributed to an increase in ruminal passage rates associated with cold adaptation. Consequently, ruminal digestion decreases and methanogens are less able to compete in such conditions (Moss et al. 2000). Furthermore, a rapid passage rate favours propionate production resulting in an expected reduction in methane production. Kennedy and Milligan (1978) reported that due to cold exposure, methane production was decreased by 30%, while the ruminal passage rate of liquid and solid phase increased by 54% and 68%, respectively. Okine et al. (1989) also observed this inverse relationship between methane production and passage rate. In their study, the methane production decreased by 29% while the fractional passage rate of particulate matter was increased 63% in steers. In addition, a decrease in acetate and an increase in propionate production due to cold exposure suggest a shift from methane to propionate production (Kennedy and Milligan 1978). On the other hand, the study by Mishra et al. (1970) indicated that

high environmental temperature reduced rumen motility. Thus, it is expected that methane production would increase at higher ambient temperatures.

In contrast to the above general relationship between ambient temperatures and methane production, Von Keyserlingk and Mathison (1993) reported that sheep housed at 4.7 ^{0}C produced 25% more methane (L/d) than those housed at 21 ^{0}C. When methane production was expressed as a percentage of digestible energy, 14% more methane was produced in the cold environment. This result was partly attributed to an 8% increase in dry matter intake at the cold temperature, and to the sheep in the study, which may not have been truly cold stressed. Similarly, Dmytruk et al. (1995) observed that higher methane production, expressed as either % of GE or DE intake, occurred at -23.1 ^{0}C than at 29.4 ^{0}C. Thus, the assumption cannot always be made that the methane production from ruminants will reduced at low ambient temperature.

2.8.3. Microbiological Considerations

It is well known that there is a close association between rumen protozoa and methanogens (Finlay et al. 1994; Tokura et al. 1997). Protozoa in the rumen are responsible for a high proportion of the hydrogen production, and provide a habitat for up to 20% of rumen methanogens (Stumm et al. 1982). Methanogens associated with protozoa could produce up to 37% of rumen methane (Finlay et al. 1994).

Defaunation, which is the elimination of protozoa from the rumen by dietary manipulation or chemical agents, has been shown to decrease rumen methane production by 20 to 50% (Van Nevel and Demeyer 1996). Reviewing the literature, Kreuzer et al. (1986) calculated that defaunation decreased energy losses through methanogenesis from 5.49 to 7.87% of gross energy intake. Whitelaw et al. (1984) observed a 50% decrease in

methane production following defaunation of cattle fed a barley-based diet. However, in some in vivo trials, the effect of defaunation on methane production was dependent on the composition of the diet. Itabashi et al. (1984) reported that defaunation had no effect with an all-hay ration, while 30 % reduction in methane production due to defaunation was noted when goats were fed the high concentrate diet.

It is assumed that there is a symbiotic hydrogen transfer between anaerobic protozoa and methanogens, which allows the protozoa to dispose hydrogen to methanogens and produce more acetate (Stumm et al. 1982). The reduced ruminal methanogenesis observed with defaunation can be attributed to factors such as a shift of digestion from the rumen to hind gut; lower rumen digestion of fibre; the loss of methanogens associated with protozoa (Van Nevel and Demeyer 1996).

Recently, Hegarty (1999) reviewed some strategies for eliminating rumen protozoa to reduce methane emissions, such as the manipulation of diet, the application of synthetic chemicals and natural compounds, as well as some potential biological agents. In addition, Klieve and Hegarty (1999) have more systematically reported that biological agents possess the potential capacity of suppressing or eliminating rumen protozoa. These biological agents include fungal pathogens, viruses, and bacteriocins. However, biological defaunating agents are not currently available for commercial use, because their efficacy has not been determined.

2.8.4. Genetic Considerations

Lal et al. (1987) observed that energy loss through methanogensis (% of GEI) was higher in Hostein-Friesian × Harian cross cattle than in Holstein-Friesian cattle fed on wheat straw-based rations. More recently, Robertson and Waghorn (2002) observed

that Dutch/US cross Holstein cows produced 8–11% less methane (% of GEI) than New Zealand Friesian cows, either when grazing or receiving a total mixed ration. This can be attributed to the fact that the indigenous breeds possess larger volume rumen as a result of the capacity to consume and utilize the poor quality forages. Thus, an increase in fractional methane losses resulting from longer rumen retention time would be expected.

Mean retention time of digesta is a highly repeatable trait (repeatability = 0.45-0.6) in sheep (Smuts et al. 1995). Thus, there is an opportunity for genetic selection of superior animals for this trait which would be expected to produce less methane. Selecting animals for a faster passage rate of feed from the rumen would reduce methane production per unit of feed ingested. Also, faster passage rate of feed affects propionate and microbial production. Therefore, selection of animals for this could have major production benefits.

Hegarty (2001) noted that the natural variation among animals in the quantity of feed eaten per unit of liveweight gain can be exploited to breed animals that consume less feed than the unselected population while achieving a desired rate of growth. To exploit such traits, the concept of Residual Feed Intake (RFI) was developed and used (Koch et al. 1963; Archer et al. 1998). The RFI, defined as actual feed intake minus the expected feed intake of each animal, is moderately heritable ($h2 = 0.39$), and is independent of the rate of gain (Arthur et al. 2001). Okine et al. (2002) calculated annual methane production from Canadian high Net Feed Efficiency (NFE) steers to be 21% lower than that for low NFE steers. Thus, selecting animals with high NFE could be a mitigation strategy to reduce methane production from ruminants.

2.9. Using Ionophores as a Strategy to Reduce Enteric Methane Production

2.9.1. Ionophores

Ionophores are lipid soluble substances that form a channel or act as a carrier in a lipid bilayer membrane to allow specific ions to move across the membrane. Their molecular weights usually range from 500-2000. The exterior of the molecule is hydrophobic, while the interior is hydrophilic and able to bind cations. In the rumen, ionophores bind to bacterial membranes where they concentrate, and to a lower degree, to protozoal membranes and feed particles. Little ionophore is free in the ruminal liquid phase (Chow et al. 1994).

Ionophores are naturally occurring compounds which are fermentation products of several actinomycetes and were originally developed as coccidiostats for poultry (Richardson et al. 1976). Since the mid-1970s, ionophores have been extensively used to manipulate rumen fermentation and thereby improve the efficiency of feed utilization or weight gain of growing ruminants (Russell and Strobel, 1989). Monensin is by far the most commonly used ionophore, but others, such as lasalocid, tetronasin, salinomycin, lysocellin, narasin, nigericin, laidlomycin, and valynomycin, either have been investigated or are used commercially.

Ionophores have additional benefits in reducing the risk of certain cattle digestive disorders (McGuffey et al. 2001), including bloat, an excess production of stable foam in the rumen, and acidosis, an accumulation of lactic acid and/or volatile fatty acids in the rumen due to an increase of rapidly fermentable carbohydrates (i.e. grain) in the diet.

It has been found that rumen bacteria resistant to one ionophore can also be resistant to other ionophores (Newbold et al. 1993). However, it is generally recognized that the use of ionophores in animal feed is not likely to have a significant impact on the transfer of the resistance from animals to human, since the action mode of ionophores differs from other major antibiotic groups, and they are not used therapeutically in humans (Russell and Houlihan 2003).

2.9.2. Mechanism of Ionophore Action

Ionophores are toxic to gram-positive bacteria, protozoa, and fungi in the rumen (Russell and Strobel 1989). This can be attributed to their capacity to penetrate into biological membranes and subsequently alter the flux of ions from and into the cell. At the membrane interface, ionophores interfere with the flux of ions either by forming cycling ion-ionophore complexes that function as ion-selective mobile carriers (e.g. monensin and lasalocid) or by creating pores that promote a less specific influx and efflux of ions (e.g. gramicidin) (Russell and Strobel 1989). However, only ionophores that act as mobile carriers have been used as feed additives.

Within this group, some ionophores such as valynomycin, work as uniporters (i.e. transport cations into the cell with no exchange for H^+). However, ionophores supplemented in cattle diets (e.g. monensin and lasalocid) normally act as antiporters (i.e. exchange H^+ cations for monovalent cations) (Fellner et al. 1997). Antiporters bind protons or metal ions (e. g. sodium and potassium), and only uncharged molecules containing either a proton or metal ion can move freely through the cell membrane (Russell and Strobel 1989). Nevertheless, the consequences of exposing bacteria cells to this group of ionophores are similar. That is, as the ionophore-mediated alterations in ion

flux progress, the activity of Na/K pump and H^+ ATPase system of the bacterial cells increases significantly in order to maintain ion balance and intracellular pH. As a result, the expenditure of energy for maintaining normal cell functions increases. This use of energy for non-growth purposes persists to the point where bacterial cells are virtually depleted of ATP, compromising the bacteria's capacity to grow and reproduce (Bergen and Bates 1984).

Since the carboxyl group of antiporters remains near the surface, and its ionization is a pH-dependent function, monensin and lasalocid are often more effective in the lower pH environment (Chow and Russell 1990). In addition, the direction of proton movement is ultimately dictated by the magnitude of ion gradients across the cell membrane, although they have different selectivities for different ions (Russell and Strobel 1989).

<u>2.9.3. Effects of Ionophores on Ruminal Microoganisms</u>

Not all rumen microorganisms are sensitive to ionophores. It is generally accepted that the sensitivities of ruminal microorganisms to ionophores are closely related to their cell wall structure (Russell and Strobel 1989). Gram-negative bacteria have an outer membrane that is impermeable to ionophores. Thus, they are resistant to ionophores. Gram-positive bacteria, which lack a protective outer membrane, are sensitive to ionophores. However, some gram-negative bacteria which actually have gram-positive-like cell wall structure are also sensitive to ionophores. For example, Chen and Wolin (1979) observed that growth of three *Butyrivibrio fibrisolvens* strains was completely inhibited by 2.5 µg/ml of monensin or lasalocid.

In vitro and in vivo studies show that monensin reduces methane production, but methanogenic bacteria are not particularly sensitive to ionophores (Van Nevel and Demeyer 1977). When mixed cultures containing monensin are incubated with H_2 and CO_2, little decrease in methane production is observed (Van Nevel and Demeyer 1977). However, monensin can inhibit H_2-producing rumen bacteria which supply methanogens with H_2 (Chen and Wolin 1979). Therefore, it seems that the decrease in methane production could be attributed to reduction in hydrogen production, which is the primary substrate of methanogenesis in the rumen.

Bergen and Bates (1984) reported that bacteria that produce lactate (e.g. *Streptococcus bovis*) were sensitive to ionophores, while bacteria that utilize lactate (e.g. *Selenomonas ruminatium and Megasphaera elsdenii*) were resistant to ionophores. This is consistent with decreases in ruminal lactate and increases in ruminal pH when ionophores are included in animal diets (Russell and Strobel 1989). Thus, the different effects of ionophores on these bacteria would be beneficial when animals are at high risk of experiencing the ruminal acidosis.

Previously isolated ammonia-producing bacteria, such as *Bacteroides ruminicola* and *Selenomonas ruminantium*, were resistant to ionophores (Bladen et al. 1961). However, Chen and Russell (1989) reported that some ammonia-producing bacteria were sensitive to ionphores. More importantly, these bacteria had 20-fold higher rates of ammonia production and were present at significant numbers in vivo. Sensitivity of these bacteria to ionophores is consistent with observations of decreased ammonia production in vitro and in vivo (Russell and Strobel 1989).

The rumen has a complex population of protozoa, and can account for up to 50% of the microbial mass (Coleman 1980). In vitro studies showed that ruminal protozoa were sensitive to ionphores (Hino 1981). The sensitivity of rumen protozoa to ionophores was also found to be dose-dependent by Wallace et al. (1981). These researchers observed no effect on protozoal population at the lowest tested dose of monensin (2mg d^{-1}) using a Rumen Simulation Technique (Rusitec) but the protozoal population decreased with increased doses of monensin (10 and 50 mg d^{-1}). In vivo studies indicated that in different feeding conditions ionophores did not necessarily decrease the numbers of protozoa in studied animals. Gyulai and Baran (1988) observed a decrease in total numbers of protozoa in lambs fed both high forage and high concentrate diets containing monensin, while some studies have reported that ruminal protozoal concentrations were unaffected by ionophores supplemented in either high forage (Dinius et al. 1976) or high concentrate diets (Leng et al. 1984). Nevertheless, protozoa in the rumen are associated with a high proportion of H_2 production, and are closely associated with methanogens by providing a habitat for up to 20% of rumen methanogens (Stumm et al. 1982). Finlay et al. (1994) reported that protozoa could account for 37% of the total CH_4 production. It is assumed that there is a symbiotic H_2 transfer between anaerobic protozoa and methanogens (Ushida and Jouany 1996). The reduced ruminal methanogenesis observed with the decrease in protozoal population can be attributed to factors such as a shift of digestion from the rumen to the hind gut (Van Nevel and Demeyer 1996) or the loss of methanogens associated with the decrease in protozoal population (Hegarty 1999).

Ruminal fungi were also sensitive to ionophores in vitro, but in vivo inhibition has not been consistent (Russell and Strobel 1989). Grenet et al. (1989) observed that

monensin had no effect on the fungal population, while Elliott et al. (1987) reported a complete elimination of fungi with the same daily dose (40 mg kg^{-1}) (DM basis).

Although there has been evidence of an adaptation response of microbes to ionophores fed to cattle (Rumpler et al. 1986; Mbanzamihigo et al. 1995), the mechanisms of ionophore resistance have not been well defined. Ionophore resistance appears to be mediated by extracellular polysaccharides that exclude ionophore from the cell membrane (Rychlik and Russell 2002). Because cattle not receiving ionophores have large populations of resistant bacteria, it appears that this trait is due to a physiological selection rather than a mutation per se (Russell and Houlihan 2003). While cross-resistance to ionophores is common, Chen and Wolin (1979) reported that some Bacteroide strains (*methanogenic and saccharolytic*) were resistant to monensin but not to lasalocid and vice versa in vitro.

2.9.4. Effects of Ionophores on Rumen Fermentation

Numerous in vivo studies have reported that ionophores increase the ratio of acetate to propionate with little effect on total VFA production, inhibit methanogenesis in the rumen, and increase non-ammonia nitrogen flow in the duodenum of treated animals (Joyner et al. 1979; Rumpler et al. 1986; Sauer et al. 1998). The effects on fermentation by mixed rumen bacteria in vitro are more variable, but usually propionate production is increased and methanogenesis is decreased, although the changes are not always significant (Richardson et al. 1976). Nevertheless, studies with various pure strains of rumen bacteria indicate that the overall effect of ionophores on rumen fermentation is probably due to shifts in the microbial population towards an ionophore-resistant organism, rather than changes to function of specific organisms (Chen and Wolin, 1979).

Ruminant animals derive most of their energy from the absorption of volatile fatty acids, such as acetate, butyrate and propionate. The conversion of feed to propionate is more efficient than the conversion to either acetate or butyrate, since propionate has higher enthalpy (Richardson et al. 1976). On the other hand, conversion of feed to acetate and butyrate results in the production of more methane, which can represent as much as 12% feed energy losses (Thornton and Owens 1981). Thus, more feed energy is available to the animal when ionophores supplemented in the animal diets.

Decreases in rumen ammonia production due to ionophore supplementation could contribute to a protein-sparing effect. Although ionophores can inhibit the growth of proteolytic ruminal bacteria, Hino and Russell (1986) observed that peptides and amino acids accumulated in the rumen of monensin-treated animals. Since ionophores also inhibit the degradation of protein hydrolysates as well as of protein, Russell and Strobel (1989) proposed that deamination rather than proteolysis is most affected by ionophores.

When ruminants are fed forages, ruminal pH usually is near neutral. However, feedlot finishing diets are high in grain and low in fiber, and ruminal pH can decrease drastically soon after feeding. These conditions favor rumen acidity and the potential for feedlot bloat. Ionophores reduce the production of lactic acid, a much stronger acid than the typical volatile fatty acids in the rumen, resulting in controlling acidity and reducing the incidence and severity of bloat (Nagaraja et al. 1982).

Although there is evidence of an adaptation response of rumen microorganisms to ionophores, no consensus exists in the literature concerning the duration of alterations in ruminal fermentation when ionophores are given or when ionophore administration

ceases. For instance, Carmean and Johnson (1990) reported that control values of methane production were restored on day 9 of ionophore feeding while Mabanzamihigo et al. (1996) observed that the effect persisted for 49 days. Potchoiba et al. (1984) reported that monensin maintained changes in propionate concentration for only 3 days after ionophore withdrawal. However, Dawson and Boling (1983) noted that the percentage of monensin-resistant bacteria remained high for 18 days after monensin withdrawal and the depression in the ratio of acetate to propionate lasted for 10 days. Although the reason is not clear, it appears that the composition of the diet fed, ionophore level supplemented, and different responses of animals to ionophores could be responsible for these discrepancies.

2.9.5. Effects of Ionophores on Growth Performance

Various ionophores have been fed to beef cattle and the overall effectiveness of these compounds has been found to be similar (Bergen and Bates 1984). With diets containing high levels of readily fermentable carbohydrates, ionophores generally reduce feed intake, but average daily gains are not decreased and feed efficiency is improved. When animals are fed high forage diets, ionophores have no effect, or slightly decrease feed intake, and average daily gain is increased, resulting in improved feed efficiency.

The potential for improved feed efficiency of feedlot cattle as a result of monensin supplementation is well documented (Goodrich et al. 1984; Stock et al. 1990). However, the magnitude of the response has been variable, ranging from nil (Zinn and Borques 1993) to greater than 18% (Bartley et al. 1979). The reason for the variable response is not certain. Factors that have been implicated include protein level in diets (Hanson and Klopfenstein 1979), diet energy density (Goodrich et al. 1984), and

microbial adaptation (Morris et al. 1990). Hanson and Klopfenstein (1979) reported steer growth response for two protein levels supplied by soybean meal (either 11.1% or 13.1%) and two levels of monensin (0 and 33 mg/kg). Monensin improved feed efficiency by 8.1% and 3.1% on the 11.1% and 13.1 % protein diet, respectively. Goodrich et al. (1984) observed that the optimum diet energy density for monensin addition was 12.2 MJ/kg of ME. As diet energy density increased above this level, feed efficiency responses decreased. Nevertheless, the beneficial effect of monensin on feed efficiency could be attributed to a combination of the energetic advantages of a higher propionate fermentation (Richardson et al. 1976), increased amounts of feed protein escaping microbial degradation (Van Nevel and Demeyer 1977), and reduced loss of carbon by methanogenesis (Rumpler et al. 1986).

2.9.6. Levels of Ionophores Used

The concentration of monensin most commonly supplemented in the cattle diets is 33 mg/kg DM, with the range from 11 to 33 mg/kg DM. According to the review by Goodrich et al. (1984), feed intake decreased as the amount of monensin in the diet increased, while the average daily gain seemed to be identical at various levels. Richardson et al. (1976) observed that molar proportion of ruminal propionic acid increased with increased level of monensin both in vitro and in vivo. This indicates an increase in efficiency of use of the feed energy and ruminants would have more energy available for metabolism from the feed consumed.

Under the regulations of Canadian Food Inspection Agency (1996), the maximum of monensin and lasalocid used as growth promoters in the beef cattle diets are 33 and 36 mg/kg dry matter, respectively. Higher feeding levels are not only illegal but

can be toxic to cattle. Potter et al. (1984) conducted a multi-dose study to determine the onset and degree of clinical signs of monensin toxicity in cattle, weighing 210 to 308 kg. Animals on the 400, 600 and 1000 mg/d monensin treatments showed mild or delayed onset of depression and diarrhea. Animals on the 2000 and 4000 mg/d treatments showed a progression of signs: anorexia, diarrhea, depression, rapid breathing, and some deaths. In addition, mixing errors and misuse of ionophores under the practical conditions have resulted in cases of cattle mortality (Potter et al. 1984).

3. MANUSCRIPT Efficacy of Ionophores in Cattle Diets for Mitigation of Enteric

Methane

3.1. Abstract

Use of ionophores in cattle diets has been proposed as a mitigation strategy for enteric methane (CH_4) emissions. The short and long term effects of feeding a single ionophore (monensin) or rotation of two ionophores (monensin and lasalocid) on enteric CH_4 emissions of beef cattle were evaluated in the present study. Thirty-six Angus yearling steers (328 ± 24.9 kg) were used over the course of the 16-week feeding trial. They were randomly assigned to six dietary treatments of six steers each. The six diets were high-forage without ionophore supplementation, high-forage with monensin supplementation, high-forage with a two-week rotation of monensin and lasalocid supplementation, high-concentrate without ionophore supplementation, high-concentrate with monensin supplementation, and high-concentrate with a two-week rotation of monensin and lasalocid supplementation, respectively. Enteric methane emissions, as measured using the SF_6 tracer gas technique, ranged from 54.7 to 369.3 L d^{-1} over the course of the study. Methane energy lost (% GEI) ranged from 1.1 to 9.2 % on the high-forage diets and from 0.9 to 8.4 % on the high-concentrate diets. Supplementing ionophores resulted in decreases ($P<0.05$) by 30% and 27% in methane emissions, expressed as L kg^{-1} DMI or % GEI, for the first two and four weeks for cattle receiving the high-concentrate and high-forage diets, respectively. No benefit related to either extent of decline in methane emissions or length of time was observed for the rotation of ionophores, compared with monensin supplementation. Total volatile fatty acid concentration of rumen fluid was not changed ($P>0.05$) as a result of ionophore addition to the diets, however, the ratio of acetate and propionate, and ammonia-nitrogen concentration in the rumen fluid were decreased ($P<0.001$) from the time ionophores

were introduced to the time they were removed from the diets. Both monensin, and rotation of monensin and lasalocid decreased (P<0.001) total ciliate protozoal populations by 82.5% and 76.8% in the first two and four weeks they were supplemented in the high concentrate and high forage diets, respectively. Original total ciliate protozoal populations were restored by the sixth week of supplementation when cattle were fed the high forage diet, and by fourth week of supplementation when cattle were fed the high concentrate diet. No significant change was observed thereafter. These data suggest that the effects of ionophores on enteric methane production are related to ciliate protozoal populations, and that ciliate protozoal populations can adapt to the ionophores presented in either high forage or high concentrate cattle diets. Furthermore, it appears that rotation of monensin and lasalocid did not avoid ciliate protozoal adaptation to ionophores.

Key words: Feedlot steers, ionophores, enteric methane, rumen fermentation, protozoa

3.2. Introduction

In recent years, there has been considerable interest in methane (CH_4) production by the ruminant animal, since it contributes to global CH_4 emissions and is involved in global warming. It has been estimated that ruminant CH_4 production is responsible for approximately 95% of total global animal and human CH_4 emissions (Johnson et al. 1991). In Canada, methane emissions from cattle production account for 97% of total enteric fermentation emissions (Janzen et al. 1999). Ninety percent of ruminant CH_4 production originates from rumen fermentation, while only 10% is produced in the hindgut (Murray et al. 1978). Therefore, considerable research effort has been devoted to find the best strategy for mitigation of enteric methane. Some strategies,

such as biotechnological strategies, for reducing CH_4 production are impractical at the present time. However, the use of ionophores as a strategy for decreasing CH_4 emission from ruminants would be economically feasible, because this feed additive is commonly used in cattle production system.

Ionophores are highly lipophilic substances which are toxic to many bacteria, protozoa and fungi through shielding and delocalizing the charge of ions, and facilitating their movement across membranes (Mathison et al. 1998). Monensin and lasalocid are two ionophores which have been extensively used to manipulate ruminal fermentation and, thereby, improve the efficiency of feed utilization (Russell and Strobel 1989). Numerous studies have reported that ionophores decrease methanogenesis, increase the ratio of propionic to acetic acid production, and decrease protein degradation to ammonia (Rumpler et al. 1986; Russell and Strobel 1989; Carmean and Johnson 1990; Sauer et al. 1998). According to the review done by Johnson and Johnson (1995), ionophore additions to beef cattle diets, particularly monensin, can decrease CH_4 production by approximately 25%. However, there are studies that show the decrease in CH_4 production is short-lived (Rumpler et al. 1986; Johnson et al. 1997). Original CH_4 emission levels were restored after a few weeks in these long-term in vivo trials. Rumpler et al. (1986) and Carmean and Johnson (1990) observed that volatile fatty acid modifications induced by ionophores were maintained as long as the ionophore was given, while methanogenesis was adapted on day 12 and on day 9, respectively. This is in contradiction with the accepted stoichiometry of rumen fermentation: an increase in propionic acid production should be related to a decrease in methanogenesis (Wolin 1960). It is worthwhile to note that the experimental animals were fed a high concentrate

diet. In contrast, Mbanzamihigo et al. (1996) studied the effect of monensin on both methanogenesis and rumen fluid volatile fatty acid for 49 days and found no evidence of adaptation for experimental animals fed a high roughage diet. Although the mechanisms of ionophore resistance have not been well defined, it is speculated that rumen microbial populations can adapt to feed ionophores, resulting in a reduction in the anti-methanogenesis effect (Russell and Houlihan 2003).

It is well known that ruminal ciliate protozoa play an important role in methane production. Recent studies indicate that some rumen methanogens live in the protozoal cytoplasm or attach to the external cell surface of ciliates, using hydrogen generated by the host ciliates to form methane (Hegarty 1999). It has been hypothesized that ionophores do not affect methanogens directly, but rather act on protozoal populations which act as a symbiotic host for methanogens. Wallace et al. (1981) reported that ruminal protozoa were inhibited by monensin in vitro, but not all workers have observed a reduction in total ruminal protozoal numbers in vivo (Leng et al. 1984). In addition, the number of studies investigating the effect of lasalocid on ruminal ciliate protozoa is limited. Dennis and Nagaraja (1986) did observe that lasalocid decreased the total protozoal numbers both in vitro and in vivo.

Cross-resistance to ionophores was expected, however, Chen and Wolin (1979) reported that some Bacteroides strains (*B. succinogenes and B. ruminicola*) were resistant to monensin but not to lasalocid and vice versa. Unfortunately, there is limited information regarding ionophore combinations, synergisms and adaptations.

The objectives of the present study were 1) to evaluate the impact of the administration of ionophores on enteric CH_4 emission, 2) to discern the duration of

ionophore-mediated suppression of CH_4 production and alterations in rumen fermentation, 3) to investigate the effect of the rotation of ionophores (monensin and lasalocid) on CH_4 emission and rumen fermentation, and 4) to assess the effects of ionophores on concentrations of ruminal ciliate protozoa in cattle fed high-forage or high-concentrate diets.

3.3. Materials and Methods

<u>3.3.1. Animals and Feeding</u>

Thirty-six Angus yearling steers averaging 328 ± 24.9 kg (mean $\pm$ SD) were used to assess the effects of ionophores on enteric methane production, rumen fermentation characteristics and rumen fluid ciliate populations. Prior to the start of the experiment, steers were split into two groups to adapt, over a six-week period, to either a high concentrate or a high forage diet. The composition (DM basis) of high forage diets was 10.8% alfalfa silage, 75.2% corn silage, 12.9% canola meal, and 1.1 % mineral mix while high concentrate diets contained 22.7% alfalfa silage, 8.3% corn silage, 67.9% barley grain, and 1.1 % mineral mix (Table 3.1). The steers were cared for according the guidelines of the Canadian Council of Animal Care (CCAC 1993).

The steers were fed once a day at 0900. All steers received a vitamin A (500 000 IU) and D_3 (75 000 IU) injection in November, 2003 and again in February, 2004, respectively. The thirty-six steers were randomly assigned to one of six dietary treatments at the start of the 16-week study. Steers were held individually in feedlot pens with individual water and feed bunks. The six diets included: high forage without ionophores (FC); high forage containing monensin (FM); high forage containing either monensin or

Table 3.1. Ingredient and chemical composition (DM basis) of diets fed to steers

Ingredient	High forage diet			High concentrate diet		
	FC	FM	FM/L	CC	CM	CM/L
Barley grain (%)	-	-	-	67.9	67.9	67.9
Canola meal (%)	12.9	12.9	12.9	-	-	-
Corn silage (%)	75.2	75.2	75.2	8.3	8.3	8.3
Alfalfa silage (%)	10.8	10.8	10.8	22.7	22.7	22.7
Mineral premix (%)	1.1	1.1	1.1	1.1	1.1	1.1
Chemical composition[z]						
DM (%)[y]	44.3	45.2	44.1	64.7	63.9	64.9
GE (kJ/g)	17.8	17.8	17.8	17.8	17.8	17.8
CP (%)	13.2	13.0	12.3	13.8	13.8	13.2
NDF (%)[y]	41.6	42.1	43.0	26.4	26.7	26.0
ADF (%)[y]	25.8	26.1	25.7	14.8	15.4	15.2
IVOMD (%)[y]	57.6	58.0	56.6	69.4	70.5	70.2
Ca (%)	0.71	0.69	0.67	0.71	0.68	0.75
P (%)	0.40	0.39	0.35	0.40	0.38	0.37
K (%)[y]	1.66	1.69	1.68	1.24	1.22	1.21
Mg (%)	0.36	0.36	0.36	0.24	0.24	0.25
Na (%)	0.12	0.14	0.13	0.13	0.11	0.10
Salt (%)	0.30	0.36	0.34	0.32	0.27	0.25
Ash (%)[y]	7.49	7.62	7.31	5.95	5.75	6.08
Monensin(mg/kg)–Calc.	-	33.0	-	-	33.0	-
Lasalocid(mg/kg)–Calc.	-	-	36.0	-	-	36.0
Monensin(mg/kg)–Actual	-	44.1	-	-	39.2	-
Lasalocid(mg/kg)–Actual	-	-	22.9	-	-	19.3

[z]n = 90 for DM analysis, n = 12 for OM, GE, IVOMD, other analyses were on composited samples by 11 weeks for nutrients and by 6 weeks for ionophores.
[y]High forage diets were significantly (P < 0.05) differ from high concentrate diets.

lasalocid which were rotated every two weeks (FM/L); high concentrate without ionophores (CC); high concentrate containing monensin (CM) and high concentrate containing either monensin or lasalocid which rotated in two weeks (CM/L), respectively. Monensin (Rumensin premix; Elanco Animal Health, Division Eli Lilly Canada Inc., Guelph, Ontario) and Lasalocid (Bovatec premix; Elanco Animal Health, Division Eli Lilly Canada Inc., Guelph, Ontario) were included in the final diets at 33 and 36 mg kg^{-1} (DM basis), respectively. The diets were fed as total mixed rations. Feed offered was adjusted to ensure 5 % orts and water was available ad libitum.

3.3.2. Experimental Layout

The experiment, which lasted 16 weeks, consisted of four experimental periods. The first period, week 1 and 2, served to establish baseline values. Animals assigned to FC, FM, and FM/L were fed the high forage diet without ionophores, FC, during this period. All other steers were fed the high concentrate diet without ionophores, CC, during this period. The second period, from week 3 to 6, served to measure any short-term effects of ionophores on rumen methane production, fermentation characteristics and ciliate protozoal populations. The third period, from week 7 to 14, served to measure any long-term effects of ionophores on rumen methane production, fermentation characteristics and ciliate protozoal populations. All ionophores were removed from the high forage and high concentrate diets in period 4, week 15 and 16, to examine the effects of ionophore withdrawal on rumen methane production, fermentation characteristics and ciliate protozoal populations.

3.3.3. Feed Sampling and Analyses

Feed and ort samples were collected weekly and dried in a forced draught oven at 60 ^{0}C for at least 48 hours to determine dry matter (DM) content. Dried feed samples were ground using a Wiley Mill fitted with a 1-mm screen. Feed samples were analyzed for crude protein (CP) using a Leco CNS 2000 analyzer as described by Bilous (1999), and GE using a Parr 1241 adiabatic bomb calorimeter. In vitro organic matter digestibility (IVOMD) was determined by the method of Tilley and Terry (1963) using bovine inoculum. Neutral detergent fibre (NDF), acid detergent fibre (ADF), ash, and minerals (Ca, P, K, Mg, and Na) were analyzed by Norwest Labs, Lethbridge, Alberta. The methods they used were the methods described by Undersander et al. (1993), method no. 973.18 (AOAC 1997), method no. 942.05(AOAC 1997), and method no. 985.01 (AOAC 1997) for NDF, ADF, ash and mineral, respectively. Ionophore concentrations were analyzed by Animal Health Laboratory, Guelph, Ontario. Monensin was determined using the method FD-DRUGS-ION04 (CFIA, 1997a) and lasalocid was determined using the method OMAF Toxi-010 (CFIA, 1997b).

3.3.4. Methane Gas Sampling and Analyses

Methane gas production of each steer was measured using the SF$_6$ tracer gas technique (Boadi et al. 2002). Permeation tubes were charged with 0.27-0.34 mg of SF$_6$. Release rates of the permeation tubes used in this study ranged from 292 to 659 ng SF$_6$ min^{-1}. Stainless steel permeation tubes containing SF$_6$ with known release rates were placed in the rumen using a speculum, a week prior to the start of the experiment. Over the course of the 16-week feeding trial, 24-h hour gas samples were collected in week 1, 3, 4, 5, 6, 8, 10, 12, 14, and 16. Background air samples were collected at each time using

similar collection apparatuses. The mean minimum and maximum ambient temperature during the SF_6 tracer gas sampling period was -36.3 and 6.4 ^0C, respectively. Following the 24-h period of collection, spheres were removed and pressure checked to identify blocked or leaking capillary systems to ensure data used represented a complete 24-h period. Spheres were then pressurized to 110 Kpa with pure N_2 to prevent sample contamination prior to analysis, and to allow injection of gas samples into the sample loop of a gas chromatograph.

The gas chromatograph (Star 3600, Varian, Mississauga, ON) fitted with an electron capture detector was used for determining SF_6, and a flame ionization detector was used for determining CH_4 concentration in the collected samples (Boadi et al. 2002). Prepared standards were used to standardize the gas chromatograph for SF_6 (20.73 ppt; Scott-Marrin Inc., Riverside, CA) and CH_4 (99.7 ppm; Supelco, Mississauga, ON) prior to sample analysis.

3.3.5. Rumen Fluid Sample Collection, Preparation and Analyses

Rumen fluid was sampled approximately at 2 hours post-feeding the day after gas collection. That is, they were collected once in period 1, four times in period 2, four times in period 3, and once in period 4. Approximately 300 ml of fluid was aspirated using a Geishauser oral probe (Geishauser 1993), and the first 100 ml of fluid was discarded to avoid saliva contamination. The remainder was divided into 2 samples: 30 ml for VFA and ammonia nitrogen concentration and 50 ml for ciliate protozoa enumeration. The pH of rumen fluid samples was immediately measured using an Accumet Basic 15 pH meter and an Accumet gel-filled polymer body combination pH electrode (Fisher Scientific, Fairlawn, NJ), calibrated with pH 4.0 and pH 7.0 buffer

solutions (Fisher Scientific, Fairlawn, NJ). Thirty ml of rumen fluid was centrifuged immediately, and then the supernatant was frozen and stored at –20° for later analyses of VFA and ammonia nitrogen.

Ammonia nitrogen concentration of rumen fluid samples was determined using the method described by Novozamsky et al. (1974). Fifty ul sample of the thawed supernatant was combined with 1.5 ml of reagent I (100 ml of alkaline phenolate + 200 ml of 0.05% sodium nitroprusside + 10 ml of 4% Na EDTA). The mixture was vortexed, and 2.5 ml of reagent II (400 ml of phosphate buffer + 100 ml of 10% NaOH) reagent was added and the contents were vortexed again. Test tubes were covered and put in darkness for 30 minutes. Thereafter, absorbance on a spectrophotometer of samples was read at 630 nm and ammonia nitrogen concentrations were calculated from regression equations of the standard curve.

Volatile fatty acid (VFA) concentration in the rumen fluid was measured using the method described by Erwin et al. (1961). Frozen rumen fluid samples were thawed at room temperature and 25% metaphosphoric acid solution was added to rumen fluid in the ratio of 1:5. The tubes were vortexed and placed in a –20 ^{0}C freezer for overnight. Thawed samples were centrifuged at 3000 RPM for 20 minutes. Approximately 2 ml of supernatant was transferred into a clean dry vial. The samples were capped and placed into the autosampler device for analysis. Concentrations of VFA were determined by gas chromatography (Model 3400 Star, Varian, Walnut Creek, CA). The injector and detector temperatures were set at 170 ^{0}C and 195 ^{0}C, and initial and final column temperatures were set at 120 ^{0}C and 165 ^{0}C, respectively. The runtime was 6 minutes followed by a 2 minutes thermal stabilization period.

The 50 ml of rumen fluid which was taken to determine ciliate protozoal populations, was mixed gently by inversion. Three 500 ul subsamples were taken from each sample, and combined with 500 ul of 20% formalin, respectively. Subsamples were stored at 4 °C until ready for ciliate protozoal counting. Ciliate protozoa were counted as described by Sambrook and Russell (2001) using a standard hemocytometer chamber, and four major genera were identified as outlined by Dehority (1993).

3.3.6. Statistical Analyses

Statistical analyses were performed using a repeated measures model (assuming autoregressive covariance structure for DMI data and spatial power model covariance structure for the rest of the data sets, respectively). All statistical analyses were run with PROC MIXED (SAS Inst. Inc., Cary, NC), with least squares means (LSMEANS) and associated standard errors reported. Statistical analysis of total ciliate protozoa and individual species was performed on the log10-transformed data, with one observation added onto actual counts to meet the requirement of SAS analysis for zero observations. Statistical analysis of correlation between CH_4 production and total ciliate protozoa was performed using PROC CORR (SAS Inst. Inc., Cary, NC). It should be mentioned that one steer, assigned to CM/L, was removed from the test in week 6 due to an unrelated illness. All data for this animal was removed. Also, contamination of spheres required that all methane emission data collected in week 14 to be removed. Methane emission data for partially filled spheres that did not meet designated criteria were not used for statistical analysis. Numbers of observations are reported in all cases. The following model was used:

$$Y_{ijk} = \mu + T_i + A_{ij} + W_k + (TW)_{ik} + e_{ijk}$$

Where Y_{ijk} = trait under consideration; μ = overall mean; T_i = dietary treatment (i=1,2,3,4,5,6); A_{ij} = animal within treatments; W_k = week (k =1...16); $(TW)_{ik}$ = treatment x week interaction; e_{ijk} = experimental error term. Means were separated at the 5% level of significance using the probability of differences (PDIFF) option.

3.4. Results and Discussion

3.4.1. General Trial Comments

Cold temperatures during winter months are common occurrences throughout Canada. Environment Canada Statistics for Winnipeg International Airport, located 20 km form the study site, reported that mean weekly ambient temperature ranges from -28.6 to 6.7 ^{0}C over the 16 weeks making up the present trial (Figure 3.1). Cold exposure can have a devastating effect on beef animal performance, as a result of high maintenance needs and faster passage rate through the gut with subsequently lowered digestibility of feed (Kennedy and Milligan 1978).

Diets were formulated to sustain different rumen microbial populations by using different ratios of cell wall (structural carbohydrate) versus starch (non-structural carbohydrate). The high forage and high concentrate diets were formulated to be iso-nitrogenous, and to ensure vitamin and mineral requirements for growing animals were met (Table 3.1). The DM and IVOMD of high forage diet were lower than that of high concentrate diet, while the NDF and ADF content of high forage diet was higher (Table 3.1). Ionophore levels measured in diet samples differed from formulated levels, with measured monensin concentrations in both diets being higher, while lasalocid concentrations measured were lower than formulated levels. Measurements were made on mixed samples. It may be that the process of mixing concentrates containing
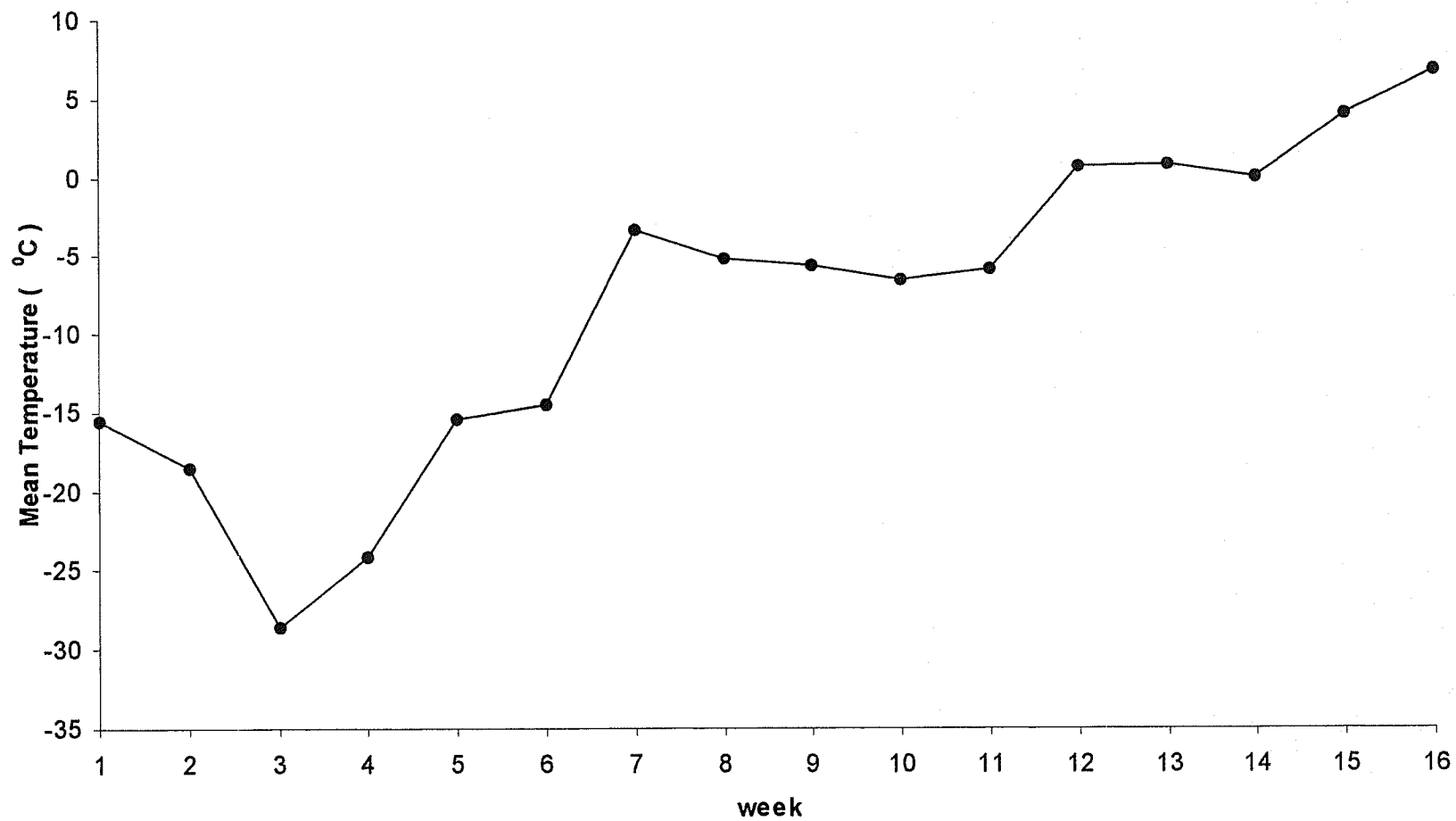
Figure 3.1. Mean weekly ambient temperatures over the course of the 16-week trial

ionophores with silage was uneven (Young and Craig 2001), or that the process of grab sampling, compositing of daily samples and subsequent subsampling contributed to an inaccurate assessment of diet ionophore content as total ionophore disappearance matched expected utilization according to formulation. As well, based on the lab values for monensin concentration for both the high forage and high concentrate diets, animals should have exhibited digestive upset (Potter et al. 1984).

The effect of diet regime on the 16-week animal intake and performance response to supplemental ionophores is shown in Table 3.2. Steers consuming the high forage diet ate 7.28 kg d^{-1} which was less ($P < 0.05$) than the 8.21 kg d^{-1} consumed with the high concentrate diet (DM basis). Animals consuming the high forage diet also had higher feed conversion rate (FCR) ($P < 0.05$) than those fed the high concentrate diet, while ADG for these diets was similar ($P > 0.05$). Similarly, Reed and Whisnant (2001) observed that animals consumed a lesser amount of high forage diet than those fed a high concentrate diet. This may be due in part to the bulk of the high forage diet. In addition, the present trial used 5% orts as a criteria for daily adjustment of feed offered which may have disadvantaged consumption of the high forage diet because animals were not consuming the larger corn silage pieces (> 5cm) once frozen.

Both monensin and rotation of monensin and lasalocid did not influence ($P > 0.05$) DM intake, ADG, and FCR of the steers consuming the high forage diet, which is consistent with other studies (Stock et al. 1990; Zinn et al. 1994). However, when animals were fed the high concentrate diet, both monensin and rotation of monensin and lasalocid decreased ($P < 0.05$) DM intake (kg d^{-1}) and improved ($P < 0.05$) FCR, while ADG was not influenced ($P > 0.05$) (Table 3.2). Similarly, in the study of Bartlet et al.

Table 3.2. Effect of diet on animal intake and performance

Parameter	High forage diet			High concentrate diet			SE
	FC	FM	FM/L	CC	CM	CM/L^z	
DM intake, kg/d	7.28^b	7.03^b	7.22^b	8.21^a	7.36^b	7.17^b	0.21
DM intake, %BW	1.84	1.87	1.87	2.09	1.94	1.91	0.09
GE intake, kJ/d	129.3^b	125.4^b	128.1^b	146.3^a	131.2^b	127.1^b	2.91
DOM intake, kg/d	3.76^c	3.65^c	3.70^c	5.18^a	4.69^b	4.58^b	0.05
CP intake, kg/d	0.90	0.86	0.88	1.09	0.98	0.95	0.09
ADG, kg/d	0.88	0.90	0.89	1.05	1.03	1.01	0.05
Feed:gain ratio	8.21^a	7.98^a	8.00^a	7.72^b	7.23^c	7.11^c	0.08

^{z}n = 6 except for CM/L which had one less animal.
^{a,b}Within a row, means with different superscripts differ.

(1979), neither monensin nor lasalocid affected the growth rate in cattle while each reduced feed intake and improved feed efficiency. Therefore, improved feed conversion efficiency seen in the present study due to ionophore supplementation is likely to be related to the reduction in DM intake, and not a direct effect on growth rate. Overall, literature reports vary on the effects of ionophore supplementation on beef cattle performance and the reason is not certain. Goodrich et al. (1984) observed that cattle consuming large amounts of ME responded more to monensin than cattle consuming less ME, and the optimum values of diet ME for monensin supplementation was 12.13 kJ/kg. The ME values for high forage and high concentrate diets used in the present trial were 9.25 and 11.17 kJ/kg, respectively.

3.4.2. Enteric Methane Production

Enteric CH_4 production ranged from 54.7 to 369.3 L d^{-1} in the course of the study. Methane energy lost (% GEI) ranged from 1.1 to 9.2 % on high forage diet and from 0.9 to 8.4 % on high concentrate diet. These values met designated criteria for growing animals according to the observations by Boadi and Wittenberg (2002) and Boadi et al. (2004b). Consistent with expectation, enteric CH_4 production by animals consuming the high concentrate diet (6.1% of GEI) was less (P < 0.05) than that by animals consuming the high forage diet (7.3% of GEI). The study by Moss et al. (1995) illustrated that the forage to concentrate ratio of the ration has an impact on CH_4 production. Van Soest (1982) indicated that a high grain diet shifts the fermentation pattern in the rumen to give rise to a more hostile environment for the methanogenic bacteria. Methane production gradually decreases with increasing amount of concentrate, which is fermented at a high rate in rumen (Ørskov et al., 1968). Thus, source of energy (non-structural carbohydrate) in the high concentrate diet

(Table 3.1) would have contributed to the lower fractional losses of methane in the present study.

Neither monensin supplementation nor rotation of monensin and lasalocid supplementation had an effect on enteric methane production, expressed as $L\ d^{-1}$ or $L\ kg\ BW^{-1}$, for the high forage or high concentrate diet. However, both monensin and rotation of monensin and lasalocid decreased ($P < 0.05$) enteric CH_4 production by 30% and 27%, whether expressed as $L\ kg^{-1}$ DMI or % GEI, for the first four weeks and for the first two weeks for high forage and high concentrate diets, respectively (Figure 3.2; Figure 3.3; Table 3.3). Enteric CH_4 emissions were restored to original levels by the third and seventh week for the high forage and high concentrate diets, respectively. The literature does include in vivo trials which demonstrate that inhibition of methanogenesis by monenson and lasalocid does not persist. Control values were restored on day 12 of ionophore feeding (Rumpler et al. 1986) and on day 9 (Carmean and Johnson 1990), in studies where the experimental animal is fed a high (70-90% DM basis) concentrate diet. Saa et al. (1993) indicated that cattle fed a high forage diet had ruminal methane production restored to original levels by day 16 with ionophore supplementation. Why ionophores failed to persistently suppress CH_4 production in these studies is not certain. These results suggested an adaptive response by microbes to ionophores supplemented in cattle diets, resulting in a reduction in the anti-methanogenesis effect (Saa et al. 1993; Russell and Houlihan 2003). In the present study, it should be noted that the duration of ionophore-mediated suppression of CH_4 production when animals were fed the high forage diet was longer than when animals were fed the high concentrate diet.
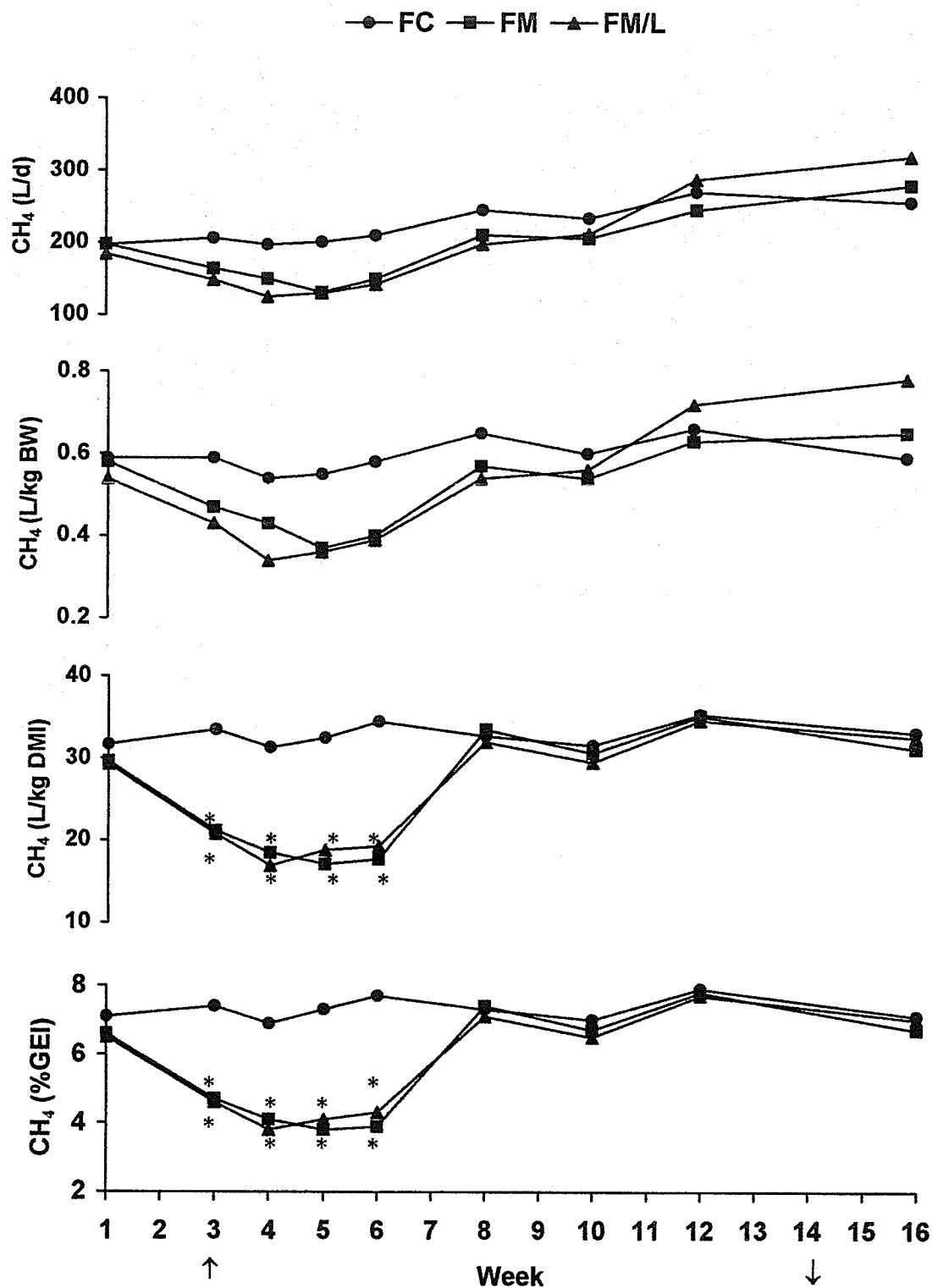
Figure 3.2. Effect of ionophores on enteric CH_4 production by steers fed the high forage diets. * Means within the same time period differ (P<0.05). Up and down arrows indicate the introduction and withdrawal of ionophores, respectively. Standard errors for CH_4 production, expressed as L/d, L/ kg BW, L / kg DMI and % GEI, are 43.08, 0.09, 5.29 and 1.17, respectively.
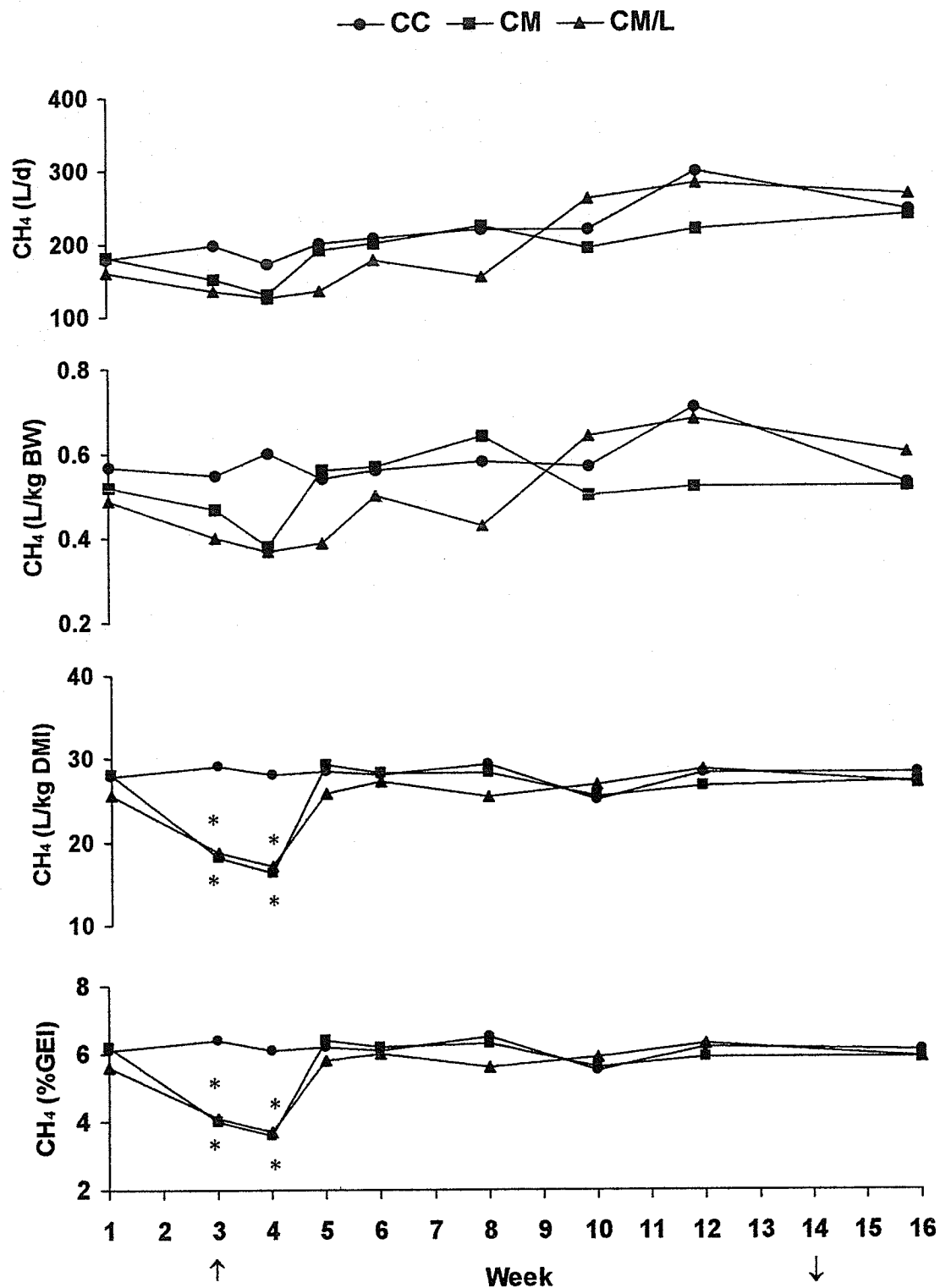
Figure 3.3. Effect of ionophores on enteric CH_4 production by steers fed the high concentrate diets. * Means within the same time period differ ($P<0.05$). Up and down arrows indicate the introduction and withdrawal of ionophores, respectively. Standard errors for CH_4 production, expressed as L/d, L/ kg BW, L / kg DMI and % GEI, are 39.92, 0.13, 5.46 and 1.20, respectively.

Table 3.3. Contrasts to show diet × week interactions for methane production

Item	1 vs 3[y]	1 vs 4	1 vs 5	1 vs 6	1 vs 8	1 vs 10	1 vs 12	1 vs 16	12 vs 16
Significance of effect[z]									
CH_4 (L kg^{-1} DMI)									
FC vs FM	*	*	*	*	NS	NS	NS	NS	NS
FC vs FM/L	*	*	*	*	NS	NS	NS	NS	NS
CC vs CM	*	*	NS	NS	NS	NS	NS	NS	NS
CC vs CM/L[x]	*	*	NS	NS	NS	NS	NS	NS	NS
CH_4 (% GEI)									
FC vs FM	*	*	*	*	NS	NS	NS	NS	NS
FC vs FM/L	*	*	*	*	NS	NS	NS	NS	NS
CC vs CM	*	*	NS	NS	NS	NS	NS	NS	NS
CC vs CM/L[x]	*	*	NS	NS	NS	NS	NS	NS	NS

[z]Significance: *$P < 0.05$; NS = not significant.

[c]1 vs 3 = contrasts of least square means in week 1 with week 3

[d]n = 6 except for CM/L which had one less animal.

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Similarly, Johnson et al. (1997) reported that the adaptive response occurred within 21 days of ionophore supplementation when cattle consumed concentrate diets, while there is no adaptive response seen up to 44 day when cattle consumed forage diets or grazed pasture. Although the reason is not clear, it appears that the adaptation of methanogenesis is related to the composition of the diet fed. Furthermore, the duration of suppression of CH_4 production (L kg^{-1} DMI or % GEI) by rotation of monensin and lasalocid was the same as for monensin singly in high forage and high concentrate diets, respectively. In a five-trial summary, Johnson et al. (1988) speculated that rotation of monensin plus tylosin and lasalocid likely prevented microbial adaptation to ionophores. It maybe that adaptation of methanogens was prevented by the presence of tylosin. Tylosin was not used in the present trial and rotation of monensin and lasalocid did not prevent adaptation.

Week to week variation of methane production for both diets can be attributed to such factors as DMI, which was influenced by variables such as weather. This has been verified for cattle fed on an ad-libitum basis, where there was a strong correlation between methane production and DMI, accounting for 64% of the variation (Boadi et al. 2002).

3.4.3. Ruminal Characteristics

The effects of diet regime on ruminal fermentation characteristics over the course of the trial are presented in Figures 3.4, 3.5 and Table 3.4. Mean rumen fluid pH was higher (P < 0.001) when animals were fed the high forage diet (6.56) as compared to the high concentrate diet (6.38). Lower ruminal pH in cattle fed high-grain diet was also observed by Hristov et al. (2001). Variability in ruminal fluid pH over the course of trial was related to
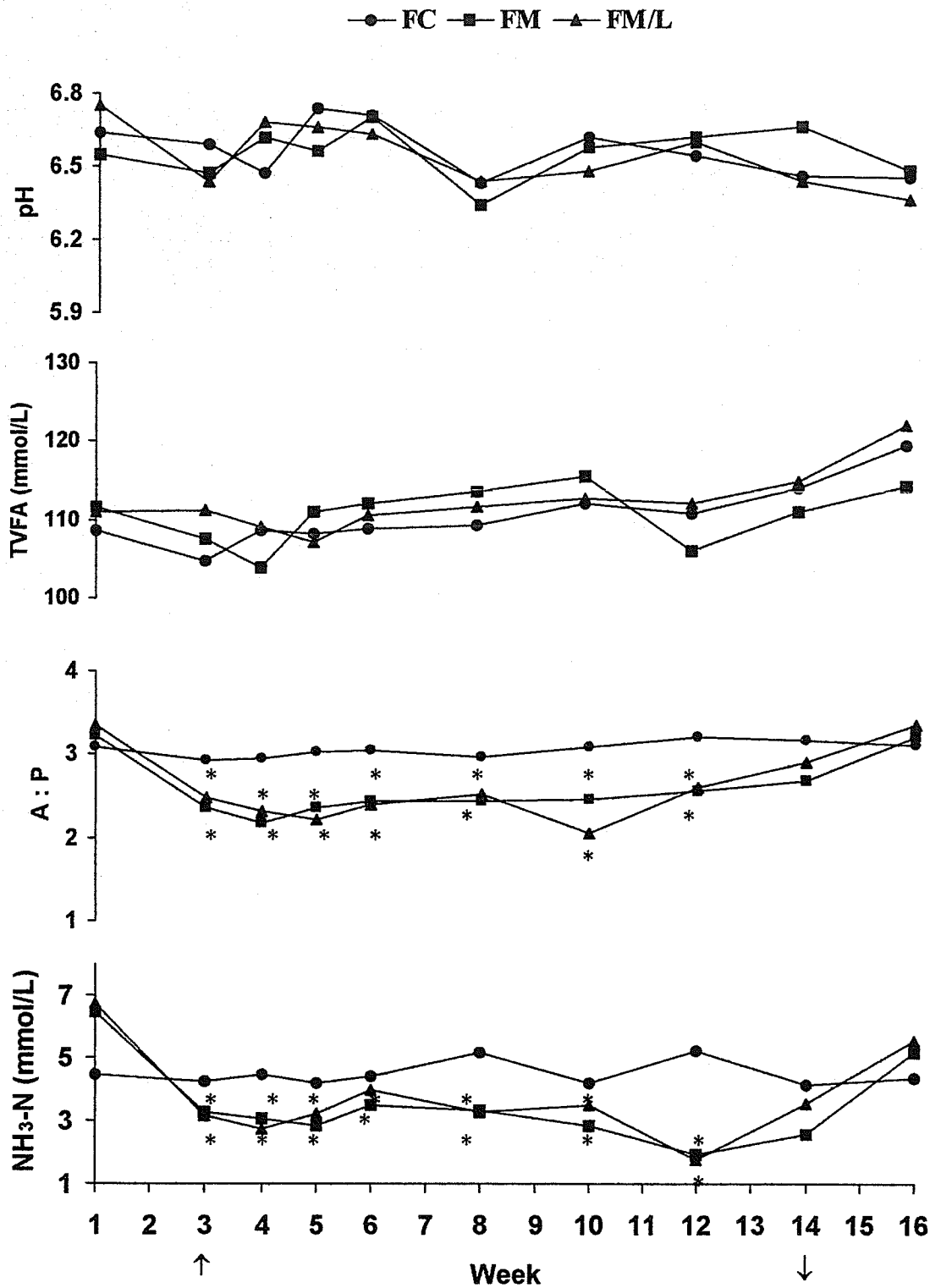
Figure 3.4. Effects of ionophores on ruminal fermentation characteristics of steers fed the high forage diets. * Means within the same time period differ (P<0.05). Up and down arrows indicate the introduction and withdrawal of ionophores, respectively. Standard errors for pH, Total VFA, ratio of acetate to propionate and NH₃-N are 0.09, 3.31, 0.11 and 0.66, respectively.
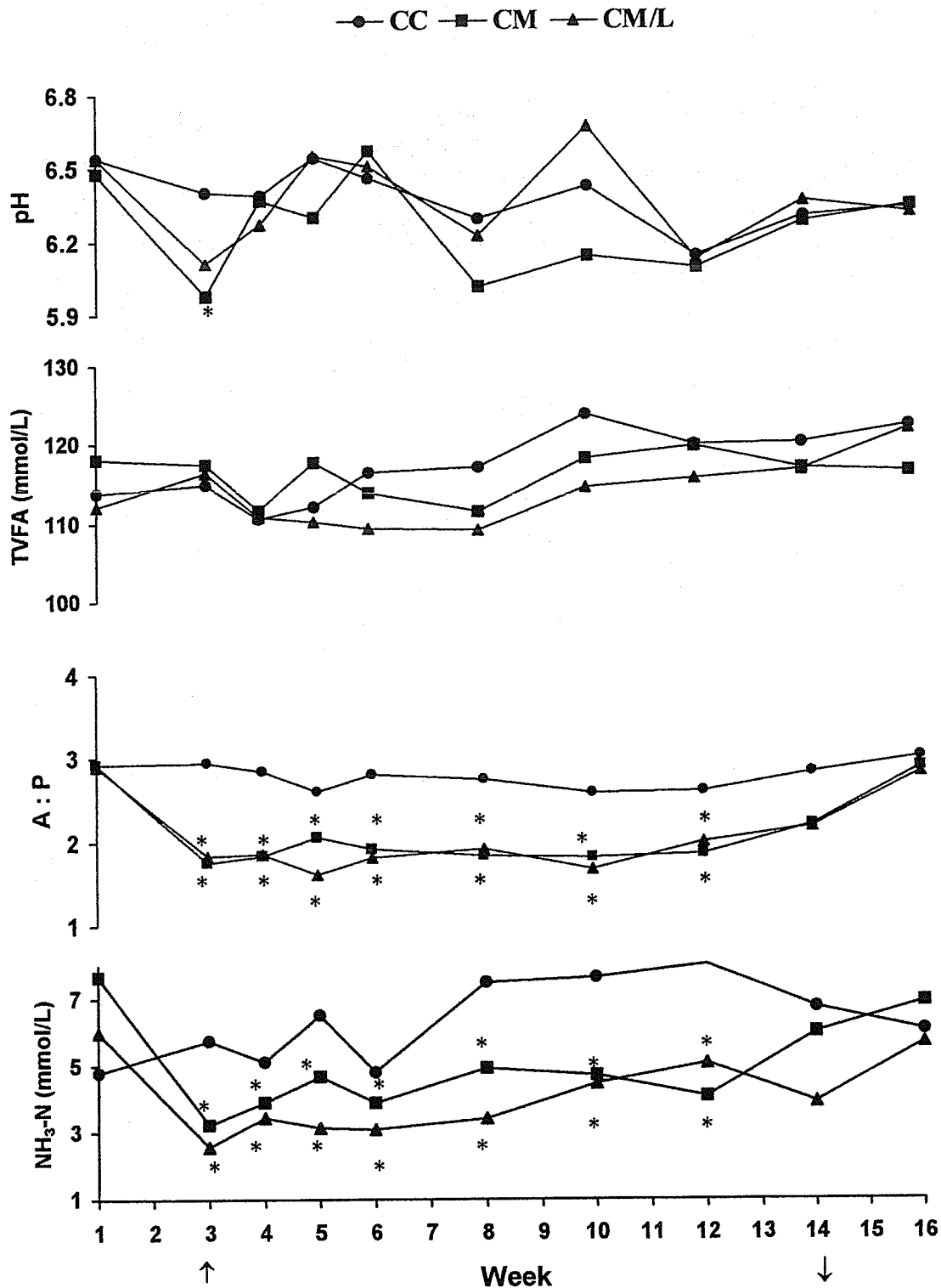
Figure 3.5. Effects of ionophores on ruminal fermentation characteristics of steers fed the high concentrate diets. * Means within the same time period differ (P<0.05). Up and down arrows indicate the introduction and withdrawal of ionophores, respectively. Standard errors for pH, Total VFA, ratio of acetate to propionate and NH_3-N are 0.10, 3.63, 0.12 and 0.73, respectively.

Table 3.4. Contrasts to show diet × week interactions for ruminal fermentation characteristics

Item	1 vs 3[y]	1 vs 4	1 vs 5	1 vs 6	1 vs 8	1 vs 10	1 vs 12	1 vs 14	1 vs 16	14 vs 16
pH										
FC vs FM	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS
FC vs FM/L	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS
CC vs CM	*	NS	NS	NS	NS	NS	NS	NS	NS	NS
CCvs CM/L[x]	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS
A : P										
FC vs FM	**	**	**	**	**	**	**	**	NS	*
FC vs FM/L	**	**	***	***	**	***	**	*	NS	*
CC vs CM	***	***	*	**	***	**	**	**	NS	*
CCvs CM/L[x]	***	***	**	***	**	**	*	**	NS	*
Ammonia										
FC vs FM	*	*	*	*	*	**	***	*	NS	*
FC vs FM/L	*	**	*	*	**	*	***	*	NS	*
CC vs CM	***	**	**	**	**	*	**	**	NS	*
CCvs CM/L[x]	**	*	**	*	***	***	**	**	NS	*

Significance of effect[z]

[z]Significance: ***P < 0.001; **P < 0.01; *P < 0.05; NS = not significant.
[y]1 vs 3 means contrasts of least square means in week 1 with week 3
[x]n = 6 except CM/L which had one less animal.

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the diet assignment. Animals on the high concentrate diet exhibited a drop ($P < 0.05$) in ruminal pH in the first week with monensin feeding as compared to animals fed the high forage diet which had no change in ruminal pH over time.

Past studies have shown that addition of ionophores increased ruminal pH of cattle consuming a high concentrate diet (Mbanzamihigo 1996), or did not influence ruminal pH of cattle consuming a high forage diet (Branine and Galylean 1990). The effect of ionophore on ruminal pH is thought to be primarily by inhibiting gram-positive bacteria, which are responsible for much of the ruminal lactate production (Russell and Strobel 1989). Reduction of ruminal lactate production could increase ruminal pH due to the low pKa (3.9) of lactate compared with the VFA (pKa 4.8) normally associated with ruminal fermentation (Russell and Hino 1985). In the present trial, with the exception of the first week of monensin supplementation in the high concentrate diet, neither monensin supplementation nor rotation of monensin and lasalocid caused a change ($P > 0.05$) in ruminal fluid pH. Although others (Rogério and Russell 1997) have observed a reduced ruminal pH with ionophore supplementation, no rationale was provided for this unusual observation. Nevertheless, Dennis et al. (1981) indicated that lasalocid was more potent than monensin in inhibiting lactic acid accumulation when cattle were fed on high-concentrate diet, while both ionophores were effective in maintaining ruminal pH and depressing lactic acid accumulation.

Total VFA concentration was not changed ($P > 0.05$) as a result of ionophore addition to either the high forage or high concentrate diets in the present trial. However, the ratio of acetate to propionate in the rumen fluid were decreased ($P < 0.001$) from the time

ionophores were introduced to the time they were removed from either the high forage or high concentrate diets (Figure 3.4; Figure 3.5; Table 3.4). Original levels of the ratio of acetate to propionate were restored within 2 weeks of ionophore withdrawal. Likewise, the ionophore-induced decrease ($P < 0.001$) in ruminal ammonia nitrogen concentration persisted throughout the entire ionophore feeding period in both the high forage and high concentrate diets. The ionophore-induced depression in ruminal ammonia nitrogen disappeared within 2 weeks after ionophore removal. Fuller and Johnson (1981) observed that both monensin and lasalocid significantly decreased ammonia production and the ratio of acetate to propionate without affecting total VFA production in vitro when added to either high concentrate or high forage substrate. Similar results were obtained by Russell and Strobel (1988). The expected in vivo effects of ionophores were confirmed with animals on either high forage (Rumpler et al. 1986) or high concentrate diets (Richardson et al. 1976). Similar withdrawal effects of ionophore on these ruminal fermentation characteristics were observed by Rogers et al. (1997), indicating no lasting effects of the ionophores on the ratio of acetate to propionate and ruminal ammonia nitrogen.

The changes in ruminal fermentation characteristics can be explained by varying sensitivities of species of rumen microorganisms to ionophores. Gram-positive bacteria are sensitive to ionophores, which influences the fermentation processes producing acetate, butyrate, lactate and ammonia. On the other hand, gram-negative bacteria, engaged in fermentation pathways associated with the production of propionate, are resistant to ionophores (Russell and Strobel 1989; Russell 1996). Proteolytic and obligate amino acid

fermenting bacteria are also sensitive to ionophores (Russell 1996). Consequently, the ratio

of acetate to propionate, and ammonia nitrogen concentration in the rumen decrease.

3.4.4. Ruminal Protozoa

Earlier literature reported that protozoa in animals fed high concentrate (> 90%)

diets were either virtually eliminated (Lyle et al. 1981) or persist at extremely low levels

(Vance et al. 1972). However, in the present trial, high levels of ruminal protozoal

concentration were observed when the animals were fed a 70% concentrate diet. This is in

agreement with several recent studies (Franzolin and Dehority 1996; Hristov et al. 2001),

which showed high concentrations of ruminal protozoa presented in diets containing 63 to

96% concentrates. Discrepancies between earlier and recent studies could be due to

differences in type of feed, number of animals sampled, or feeding regime. In addition, it

seems that diets containing between 40 and 60 % concentrate would support maximal

protozoan numbers, and even the depression in ruminal pH of cattle fed up to 75%

concentrate does not impair ruminal protozoal growth (Franzolin and Dehority 1996).

Both monensin, and rotation of monensin and lasalocid decreased (P < 0.001) total

ciliate protozoal populations in the first two and first four weeks they were supplemented in

the high concentrate and high forage diet, respectively. However, original total ciliate

protozoal populations were restored by the fourth and sixth week of supplementation in the

high concentrate and high forage diets, respectively (Figure 3.6; Figure 3.7; Table 3.5). No

significant difference due to the diets was observed thereafter. In contrast to the present trial,

some studies have reported that ruminal protozoal concentrations were unaffected by
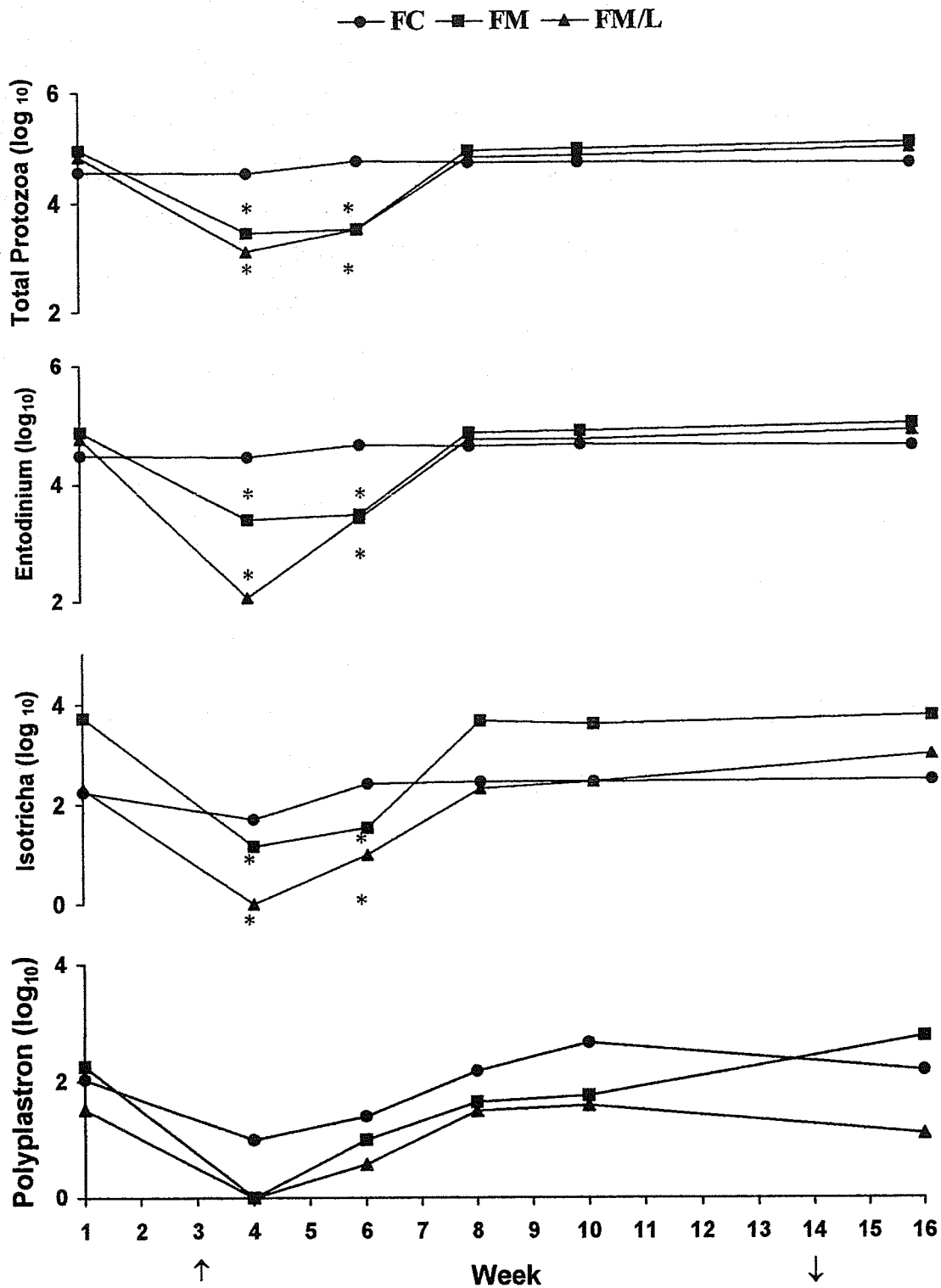
Figure 3.6. Effects of ionophores on ruminal ciliate of steers fed the high forage diets. * Means within the same time period differ (P<0.05). Up and down arrows indicate the introduction and withdrawal of ionophores, respectively. Standard errors for totoal protozoa, entodinium, isotricha and polyplastron are 0.31, 0.33, 0.55 and 0.64, respectively.
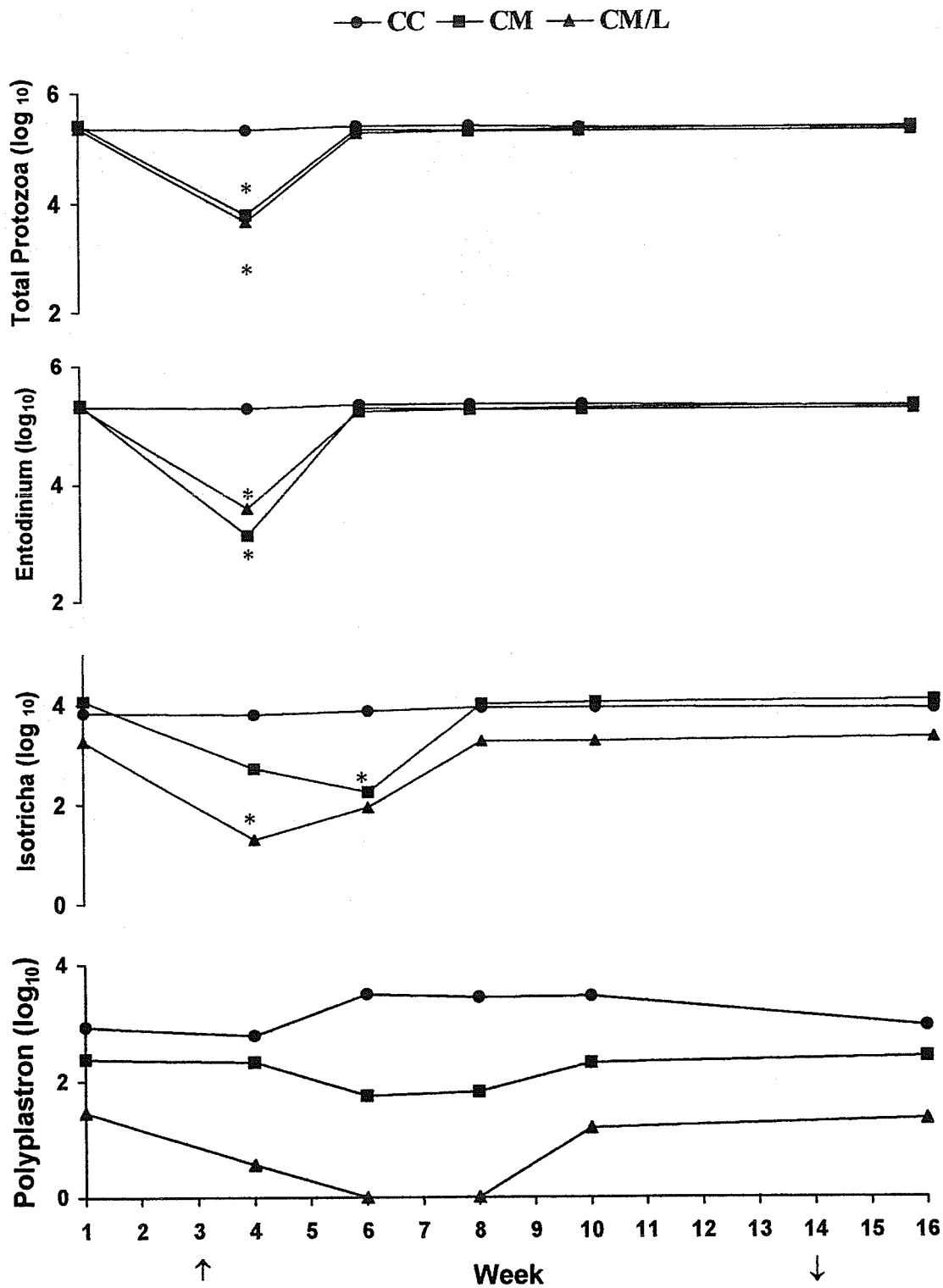
Figure 3.7. Effects of ionophores on ruminal ciliate of steers fed the high concentrate diets. * Means within the same time period differ (P<0.05). Up and down arrows indicate the introduction and withdrawal of ionophores, respectively. Standard errors for totoal protozoa, entodinium, isotricha and polyplastron are 0.34, 0.36, 0.60 and 0.70, respectively.

Table 3.5. Contrasts to show diet × week interactions for protozoal populations

Item	Significant of effect[z]				
	1 vs 4[y]	1 vs 6	1 vs 8	1 vs 10	1 vs 16
Total protozoa					
FC vs FM	*	**	NS	NS	NS
FC vs FM/L	**	*	NS	NS	NS
CC vs CM	**	NS	NS	NS	NS
CCvs CM/L[x]	**	NS	NS	NS	NS
Entodinium spp.					
FC vs FM	*	*	NS	NS	NS
FC vs FM/L	***	*	NS	NS	NS
CC vs CM	**	NS	NS	NS	NS
CCvs CM/L[x]	*	NS	NS	NS	NS
Isotricha spp.					
FC vs FM	**	*	NS	NS	NS
FC vs FM/L	*	*	NS	NS	NS
CC vs CM	*	NS	NS	NS	NS
CCvs CM/L[x]	*	NS	NS	NS	NS

[z]Significance: ***$P < 0.001$; **$P < 0.01$; *$P < 0.05$; NS = not significant.
[y]1 vs 4 = contrasts of least square means in week 1 with week 4
[x]n = 6 except for CM/L which had one less animal (n = 5)

ionophores supplemented in either high forage (Dinius et al. 1976) or high concentrate diets (Leng et al. 1984). However, in vitro studies by Wallace et al. (1981), and Hino and Russell (1986) showed that monensin decreased numbers of protozoa by 5 and 35%, while in vivo studies by Poos et al. (1979) demonstrated that reductions in protozoal concentrations due to monensin supplementation could be 64% of the control counts. Dennis and Nagaraja (1986) observed that monensin and lasalocid at all concentrations significantly decreased the total protozoal numbers when either ground corn or alfalfa hay was as substrate. Likewise, Gyulai and Baran (1988) reported that feeding 40 mg per day of monensin in either roughage or concentrate diets significantly decreased ruminal protozoal numbers in lambs.

It is recognized that ruminal ciliate protozoa are inhibited by initial ionophore exposure but subsequently develop resistance. The study by Rogers et al. (1997) shows that reductions in total ruminal protozoal numbers due to monensin supplementation decreased with time, in animals fed a high concentrate diet. They also reported that the effect of monensin on the protozoal population was species-dependent. *Entodinium* was inhibited while *Isotricha* numbers were increased after monensin addition. Dennis and Nagaraja (1986) observed that reduction in total protozoal numbers only persisted in the first two weeks of monensin supplementation when cattle were fed a high forage diet. In a subsequent in vitro study, they also indicated that ruminal protozoa adapted to monensin were resistant to lasalocid, while protozoa adapted to lasalocid seemed to be less resistant to monensin.

3.5. Conclusions

The decrease in methane production due to ionophore supplementation appears to be short-lived when cattle are fed either high forage or high concentrate diets. Although total

VFA concentration was not changed, the modification of the ratio between propionate and acetate concentration, and ammonia nitrogen concentration persisted as long as the ionophores were supplemented in either high forage or high concentrate diet. From a stoichiometry point of view, short-lived depression in enteric methane production contradicts with altered long-term ruminal VFA patterns. The data suggest that the effects of ionophores on enteric methane production are related to ciliate protozoal populations. Also, the data indicate that ruminal ciliate protozoal populations can adapt to the ionophores presented in either high forage or high concentrate cattle diets, and the high concentrate diet induces a more rapid adaptation to the ionophore. Furthermore, it appears that rotation of monensin and lasalocid does not prevent ciliate protozoal adaptation to ionophores.

3.6. Acknowledgements

4. GENERAL DISCUSSION

In feedlot cattle, monensin reduces feed intake while the rate of body weight gain is unaffected, resulting in improved efficiency of feed utilization (Russell and Strobel 1989). In the present study, when steers were fed high concentrate diets, both monensin and rotation of monensin and lasalocid decreased feed intake ($P < 0.05$) and improved feed conversion ($P < 0.05$). The improved FCR appeared to be a function of improved utilization of dietary energy (Morris et al. 1990), since decreased DMI and improved FCR were conjunct with absence of expected increased ADG. With high forage diets, neither monensin or rotation of monensin and lasalocid influenced ($P > 0.05$) ADG, DMI and FCR. This could be attributed to lower energy density in the high forage diet, compared with that in the high concentrate diet. Goodrich et al. (1984) observed that the optimum diet energy density for monensin supplementation was 12.13 kJ/kg of ME. As diet energy density decreased below this level, feed conversion response decreased. In the present study, the ME values for high forage and high concentrate diets were 9.25 and 11.17 kJ/kg, respectively.

It is interesting to note that monensin supplemented in the high concentrate diet caused a decline ($P < 0.001$) in ruminal pH while rotation of monensin and lasalocid did not influence ($P > 0.05$) ruminal pH of cattle consuming the high concentrate diet. This could be attributed to potency of lasalocid in inhibiting lactic acid accumulation when cattle were fed high concentrate diets (Dennis et al. 1981). In addition, Schelling (1984) and Zinn et al. (1994) indicated that monensin could decrease ruminal solids and liquid dilution rate, and thereby a decline in ruminal pH would be expected.

The effects of monensin or rotation of monensin and lasalocid on total VFA concentration and ratio of acetate to propionate were persistent and in agreement with abundant literature available (Richardson et al. 1976; Mbanzamihigo et al. 1996; Rogers et al. 1997). The shift in fermentation pattern to propionate can be explained by the effect of ionophores on bacteria, as shown by experiments carried out on pure cultures: bacteria which produce acetic acid and butyric acid as main end products are susceptible, while bacteria producing propionate and succinate are resistant (Russell 1996). Likewise, ionophore inhibits ammonia-producing ruminal bacteria (Chen and Russell 1989). As a consequence, monensin or rotation of monensin and lasalocid had a highly negative effect ($P < 0.001$) of the concentration of NH_3-N in the rumen as long as they were included in the animal diets.

Van Nevel and Demeyer (1977) concluded that the inhibition in methane production was not the result of a direct effect of ionophores on methanogens, but due to inhibition of the microbes producing hydrogen, the most important substrate for methanogens. This was confirmed by observations that pure strains of *Methanobacterium ruminatium* were not or were only slightly inhibited by monensin or lasalocid (Chen and Wolin 1979). Experiments with different pure strain of rumen bacteria and monensin and lasalocid indicated that the overall effect on rumen fermentation is probably due to shifts in the microbial population towards ionophore-resistant organisms, which tend to produce more propionate (Chen and Wolin 1979). With an increase in the molar proportion of propionate, the molar proportions of acetate are reduced (Baker 1997). Acetate production results in methane production, while propionate formation serves as a competitive pathway for H_2 use in the rumen. As expected, monensin or rotation of

monensin and lasalocid resulted in decreases (P < 0.05) in methane emissions, expressed

as L kg^{-1} DMI or % GEI, when cattle were fed either the high-concentrate or high-forage

diets. However, consistent with previous studies (Rumpler et al. 1986; Saa et al. 1993;

Johnson et al. 1997), inhibition of enteric methane production by ionophores did not

persist. From a stoichometric point of view, this conflicted with the altered rumen VFA

patterns with ionophores which persisted in the present study.

The data obtained in the present study suggested that reductions in methane

production due to ionophore supplementation were more closely related to reductions in

the rumen protozoa population. Since the duration of inhibition in the protozoa

population due to ionophore supplementation was matched with the duration of

depression in methane production when cattle were fed the high forage or high

concentrate diets. Furthermore, coefficient of correlation between CH_4 production

(%GEI) and total ciliate protozoa in week 4 is 0.35 (P = 0.16), while it is − 0.29 (P =

0.18) in week 10. It has been documented that methanogens living in a symbiotic

relationship with protozoa can account for about 40% of rumen methane emissions

(Hegarty, 1999), and defaunation results in reductions in emission of about 20-50%

(Kreuzer et al. 1986). Tokura et al. (1997) observed that the greatest number of

methanogens associated with protozoa occurred after feeding. The authors reported 10-

100 times the methanogens attachment per protozoa in most measurement after feeding

compared with before feeding. This difference may reflect protozoa being significant

hydrogen donor. Therefore, it appeared that decreases in methane production were due to

a decline in hydrogen production, the primary substrate of methanogenesis in the rumen.

Also, since H_2 is one of the major end products of fermentation by fungi (Wallace and

Joblin 1985), their elimination or decline in population would contribute to reductions in methane production. Although in vivo inhibition has not been confirmed one, in vitro study did show that ruminal fungi were sensitive to ionophores (Russell and Strobel 1989).

Development of rumen bacterial resistance to ionophores has been documented (Russell and Strobel 1989). A recent study indicates that ionophore resistance is mediated by extracellular polysaccharides (Russell and Houlihan 2003). However, at this time, it is difficult to explain why the high concentrate diet caused a more rapid adaptation than did the high forage diet in the present trial.

Although the majority of CH_4 production occurs in the rumen, a large amount of CH_4 may be produced in the hindgut. Murray et al. (1978) reported that CH_4 produced in the hindgut accounted for 6 to 14% of total CH_4 production. Ionophores can equally control CH_4 production in the hindgut (Mbanzamihigo et al. 1996) via acetate to propionate ratio change.

Since many external factors (feed, mud, environment, animals) can impact the success of the gas collection procedure, more animal and more sampling days are required to use SF_6 tracer gas technique. In addition, variation in CH_4 production (L/d) is great (11.6-17.2%) in the present study, which is in agreement with other reports (Ulyatt et al. 1999; Boadi et al. 2002), suggesting that more animals per treatment and sufficient collection days per animal are required to minimize variation and to detect treatment differences in experiments. Careful attention should be taken in gas collection procedure, such as sphere cleaning and vacuuming, to avoid SF_6 contamination.

Numerous studies have reported effects of ionophores, more specifically monensin, in animal diets on performance and ruminal fermentation pattern. However, little information regarding ionophores and animal metabolism is available. Thus, understanding of the effects of ionophores in ruminant metabolism is needed in future research.

5. CONCLUSIONS

It can be concluded that:

a) Ionophore supplementation had no effect on DMI, FCR and ADG when cattle were fed the high forage diets, while supplementation improved feed efficiency and decreased DMI of cattle consuming the high concentrate diets.

b) The decreases in enteric methane production due to ionophore supplementation were short-lived when cattle were fed either the high forage or high concentrate diets.

c) Modification of the ratio of acetate to propionate, and ammonia nitrogen persisted as long as ionophores were supplemented in either high forage or high concentrate diets.

d) The high concentrate diets induced a more rapid adaptation to ionophores.

e) Rotation of monensin and lasalocid did not avoid ciliate protozoal adaptation or maintain low methane emissions.

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7. APPENDIX

Data conversion and calculations of gas production rates

1. Equation used to calculate CH_4 production (liters min^{-1})

Methane production (liters min^{-1}) = SF_6 release rate (liters min^{-1}) * Ratio [CH_4 (ng kg^{-1}) / SF_6 (ng kg^{-1})]

a) Conversion of parts per million (ppm) to parts per trillion (ppt) or ng kg^{-1}: Multiplied by 1×10^6

b) Conversion of ng min^{-1} of permeation tube release rate to liters min^{-1}: divided by 6.602×10^9

c) Conversion of liters min^{-1} to liters per day: Multiplied by 1440 mins (60×24 hr; or adjusted to exact sampling duration)

2. 1 liter CH_4 = 0.716 g CH_4
   1 liter SF_6 = 6.602 g SF_6
   1 liter CH_4 = 39.54 kJ
   1 g CH_4 = 55.23 kJ

3. To calculate CH4 production (% of GEI)

   a) CH_4 production (kg y^{-1}) to CH_4 (liter day^{-1}): Multiply by 3.84

   b) CH_4 production (liter day^{-1}) to CH_4 (g day^{-1}): Multiply by 0.716

   c) CH_4 production (liter day^{-1}) to caloric value: Multiply by 39.57 kJ $litre^{-1}$

   d) CH_4 production (kJ $liter^{-1}$) to kJ g^{-1}: Divide by 0.716

   e) CH_4 production (%GEI) = [CH_4 kJ d^{-1} / (GEI (kJ d^{-1})] * 100

Calibration of permeation tube (No.18) SF$_6$ release rates during incubation at 39 ^{0}C

Empty tube weight: 17.3724 g; Filled tube weight: 17.7149 g; SF6 in tube: 0.3425 g				
Date	Incubation time (mins)	Tube weight (g)	Amount of SF$_6$ Released (g)	Release rate (ng m^{-1})
Nov. 6, 2003	11109	17.68916	0.006994	629.61
Nov. 13, 2003	10722	17.68179	0.007372	684.35
Nov. 20, 2003	10119	17.67524	0.006555	647.78
Nov. 27, 2003	10019	17.66824	0.007001	698.65
Dec. 5, 2003	11065	17.66104	0.007197	650.39
Dec. 12, 2003	10585	17.65407	0.006976	659.02
Dec. 19, 2003	10070	17.58662	0.006745	669.81

Change in canister pressure over 24-hr period
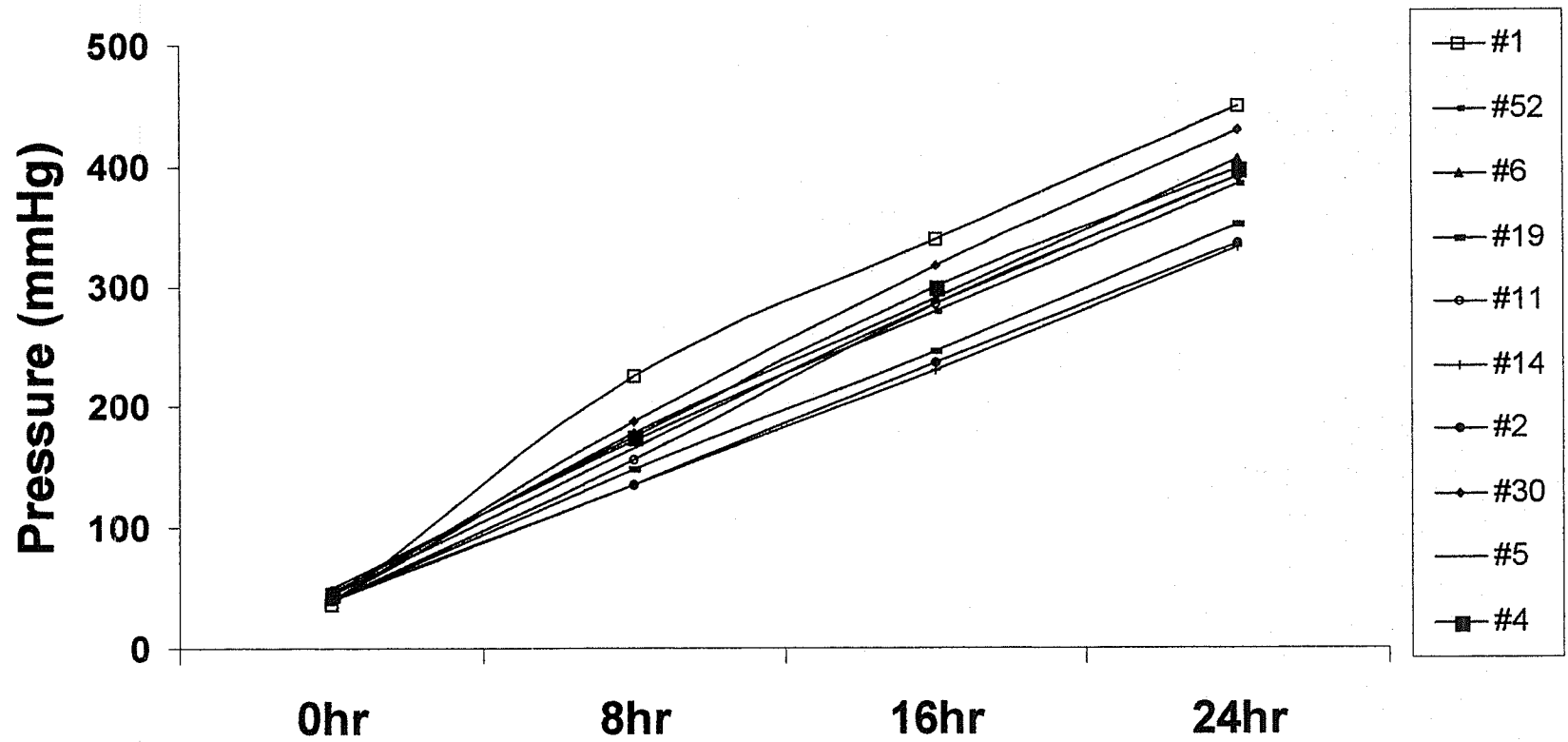
Enteric methane production of steers fed high forage or high concentrate diets

Variable	Sampling week									Mean[y]	SE	Significance of effect[z]		
	1	3	4	5	6	8	10	12	16			Diet	Week	D × W
CH$_4$ (L d^{-1})												NS	***	NS
FC	197.2	206.1	196.9	200.9	209.9	245.3	233.8	269.8	255.5	223.9	11.60			
FM	198.1	163.9	149.8	130.7	149.4	210.9	206.0	245.3	279.0	192.6	13.07			
FM/L	184.1	148.4	125.0	130.2	142.4	197.8	211.8	287.4	318.9	194.0	12.64			
CC	179.6	197.2	172.8	199.4	208.0	219.5	217.5	298.5	243.5	215.1	12.17			
CM	181.2	150.6	129.7	189.7	200.0	222.3	193.5	217.6	236.7	191.3	13.72			
CM/L[x]	160.7	134.7	124.8	133.8	175.7	154.4	261.5	280.3	264.7	187.8	17.20			
CH$_4$ (L kg^{-1} BW)												NS	**	NS
FC	0.59	0.59	0.54	0.55	0.58	0.65	0.60	0.66	0.59	0.60	0.033			
FM	0.58	0.47	0.43	0.37	0.40	0.57	0.54	0.63	0.65	0.52	0.037			
FM/L	0.54	0.43	0.34	0.36	0.39	0.54	0.56	0.72	0.78	0.52	0.036			
CC	0.57	0.55	0.60	0.54	0.56	0.58	0.57	0.71	0.53	0.58	0.035			
CM	0.52	0.47	0.38	0.56	0.57	0.64	0.50	0.52	0.52	0.52	0.040			
CM/L[x]	0.49	0.40	0.37	0.39	0.50	0.43	0.64	0.68	0.60	0.50	0.037			
CH$_4$ (L kg^{-1} DMI)												**	***	*
FC	31.7	33.5	31.3	32.5	34.5	32.7	31.6	35.3	33.1	32.9	1.51			
FM	29.6	21.2	18.5	17.1	17.7	33.5	30.6	35.1	31.1	26.0	1.68			
FM/L	29.3	20.8	16.9	18.8	19.3	32.0	29.5	34.6	32.5	26.0	1.63			
CC	27.8	29.0	28.0	28.5	28.0	29.2	25.1	28.3	28.3	28.0	1.58			
CM	28.0	18.1	16.2	29.2	28.3	28.3	25.5	26.7	27.2	25.3	1.76			
CM/L[x]	25.6	18.8	17.1	25.9	27.2	25.5	26.8	28.6	27.1	24.7	1.66			
CH$_4$ (% GEI)												**	***	*
FC	7.1	7.4	6.9	7.3	7.7	7.3	7.0	7.9	7.1	7.3	0.33			
FM	6.6	4.7	4.1	3.8	3.9	7.4	6.7	7.8	6.7	5.8	0.36			
FM/L	6.5	4.6	3.8	4.1	4.3	7.1	6.5	7.7	7.0	5.7	0.35			
CC	6.1	6.4	6.1	6.2	6.1	6.5	5.5	6.2	6.1	6.1	0.34			
CM	6.2	4.0	3.6	6.4	6.2	6.3	5.6	5.9	5.9	5.5	0.38			
CM/L[x]	5.6	4.1	3.7	5.8	6.0	5.6	5.9	6.3	5.9	5.4	0.36			

[z]Significance: ***P < 0.001; **P < 0.01; *P < 0.05; NS = not significant.
[y]Least squares means with associated SE.
[x]n=6 except for CM/L which had one less animal.

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Ruminal fermentation characteristics of steers fed high forage or high concentrate diets

Variable	1	3	4	5	6	8	10	12	14	16	Mean[y]	SE	Diet	Week	D × W
pH													***	***	*
FC	6.64	6.59	6.47	6.74	6.71	6.43	6.62	6.54	6.46	6.45	6.56	0.029			
FM	6.55	6.47	6.62	6.56	6.70	6.34	6.58	6.62	6.66	6.48	6.56	0.029			
FM/L	6.75	6.44	6.68	6.66	6.63	6.44	6.48	6.60	6.44	6.36	6.55	0.029			
CC	6.54	6.40	6.39	6.54	6.46	6.29	6.42	6.14	6.30	6.33	6.38	0.029			
CM	6.48	5.98	6.37	6.30	6.57	6.01	6.14	6.09	6.28	6.34	6.24	0.029			
CM/L[x]	6.54	6.11	6.27	6.55	6.51	6.22	6.67	6.12	6.36	6.31	6.34	0.031			
Total volatile fatty acids mmol/L													NS	***	NS
FC	108.6	104.8	108.7	108.3	108.9	109.3	112.0	110.7	114.0	119.5	110.5	1.80			
FM	111.7	107.6	103.9	110.9	112.1	113.5	115.5	106.0	110.9	114.2	110.6	1.80			
FM/L	111.0	111.2	109.0	107.1	110.5	111.7	112.7	112.1	114.9	122.0	112.2	1.80			
CC	113.9	114.8	110.6	112.1	116.5	117.1	123.7	119.9	120.1	122.2	117.1	1.80			
CM	118.1	117.4	111.7	117.6	113.8	111.5	118.1	119.7	116.9	116.4	116.1	1.80			
CM/L[x]	112.1	116.5	110.8	110.1	109.3	109.1	114.4	115.6	116.7	121.7	113.6	1.97			
Ratio of acetate to propionate													***	***	***
FC	3.09	2.93	2.94	3.02	3.04	2.96	3.09	3.20	3.16	3.11	3.06	0.036			
FM	3.22	2.36	2.17	2.35	2.44	2.44	2.46	2.56	2.68	3.21	2.59	0.036			
FM/L	3.36	2.48	2.31	2.21	2.39	2.52	2.05	2.61	2.90	3.35	2.62	0.036			
CC	2.93	2.95	2.85	2.61	2.81	2.74	2.59	2.60	2.83	3.00	2.79	0.036			
CM	2.92	1.76	1.84	2.05	1.92	1.83	1.81	1.86	2.19	2.89	2.11	0.036			
CM/L[x]	2.91	1.83	1.86	1.61	1.82	1.91	1.67	2.00	2.17	2.82	2.06	0.040			
Ammonia nitrogen mmol/L													***	***	***
FC	4.46	4.27	4.46	4.22	4.44	5.18	4.18	5.21	4.14	4.37	4.49	0.21			
FM	6.48	3.26	3.05	2.87	3.50	3.31	2.82	1.92	2.59	5.16	3.49	0.21			
FM/L	6.76	3.16	2.74	3.21	3.98	3.30	3.50	1.76	3.56	5.57	3.76	0.21			
CC	4.78	5.75	5.12	6.51	4.81	7.49	7.64	8.04	6.74	6.06	6.29	0.21			
CM	7.66	3.24	3.91	4.67	3.89	4.92	4.70	4.07	6.00	6.91	5.00	0.21			
CM/L[x]	5.99	2.57	3.44	3.13	3.09	3.42	4.46	5.06	3.90	5.69	4.07	0.23			

[z]Significance: ***P < 0.001; *P < 0.05; NS = not significant.
[y]Least squares means with associated SE.
[x]n = 6 except for CM/L which had one less animal.

Total and individual species of ruminal ciliate protozoa[z] in steers

Variable			Sampling week				Mean	SE	Significance of effect[y]		
	1	4	6	8	10	16			Diet	Week	D×W
Total									**	***	**
FC	40.63	44.67	60.13	57.53	64.47	62.67	55.02				
	(4.53)	(4.50)	(4.72)	(4.69)	(4.70)	(4.68)	(4.64)	(0.153)			
FM	89.77	12.47	13.70	84.67	93.87	109.53	67.33				
	(4.94)	(3.43)	(3.49)	(4.92)	(4.96)	(5.03)	(4.46)	(0.153)			
FM/L	69.57	8.77	13.57	66.23	71.77	88.23	53.02				
	(4.83)	(3.09)	(3.48)	(4.79)	(4.81)	(4.94)	(4.32)	(0.153)			
CC	249.13	240.30	270.80	281.97	278.10	242.77	260.51				
	(5.34)	(5.33)	(5.38)	(5.39)	(5.36)	(5.31)	(5.35)	(0.153)			
CM	299.20	48.43	243.67	221.33	253.23	253.77	219.94				
	(5.40)	(3.78)	(5.33)	(5.28)	(5.33)	(5.34)	(5.08)	(0.153)			
CM/L[x]	314.00	35.84	267.72	269.08	280.76	273.08	240.08				
	(5.36)	(3.65)	(5.27)	(5.28)	(5.29)	(5.30)	(5.02)	(0.167)			
Entodinium									***	***	**
FC	35.53	39.47	51.50	49.47	57.23	54.97	48.03				
	(4.47)	(4.44)	(4.65)	(4.62)	(4.64)	(4.61)	(4.57)	(0.153)			
FM	78.77	10.43	13.77	72.80	80.47	96.01	58.72				
	(4.88)	(3.38)	(3.47)	(4.85)	(4.89)	(4.98)	(4.41)	(0.153)			
FM/L	60.47	7.33	11.80	56.90	62.23	77.20	45.99				
	(4.76)	(2.06)	(3.42)	(4.72)	(4.74)	(4.87)	(4.10)	(0.153)			
CC	230.30	222.47	247.90	257.97	258.53	223.10	240.04				
	(5.31)	(5.29)	(5.34)	(5.35)	(5.33)	(5.28)	(5.32)	(0.153)			
CM	271.31	41.47	222.87	201.00	228.57	230.90	199.35				
	(5.35)	(3.13)	(5.29)	(5.24)	(5.28)	(5.30)	(4.93)	(0.153)			
CM/L[x]	288.70	19.08	244.80	246.56	257.16	248.16	217.41				
	(5.32)	(3.58)	(5.21)	(5.24)	(5.24)	(5.26)	(4.98)	(0.167)			
Isotricha									**	***	*
FC	1.57	1.43	2.80	2.90	2.97	3.33	2.50				
	(2.23)	(1.70)	(2.41)	(2.42)	(2.42)	(2.45)	(2.27)	(0.379)			
FM	5.67	1.33	0.77	4.67	4.00	5.87	3.72				
	(3.73)	(1.16)	(1.51)	(3.66)	(3.57)	(3.76)	(2.90)	(0.379)			
FM/L	2.33	NF[e]	0.33	2.10	3.13	3.80	1.95				
	(2.31)	(0)	(0.99)	(2.30)	(2.42)	(2.98)	(1.83)	(0.379)			
CC	8.10	6.33	7.30	8.67	9.10	8.93	8.07				
	(3.83)	(3.77)	(3.84)	(3.91)	(3.92)	(3.89)	(3.86)	(0.379)			
CM	15.33	1.67	2.23	13.20	14.33	14.00	10.13				
	(4.07)	(2.70)	(2.24)	(3.99)	(4.02)	(4.05)	(3.51)	(0.379)			
CM/L[x]	11.60	0.68	1.32	10.40	11.88	12.56	8.07				
	(3.26)	(1.29)	(1.94)	(3.24)	(3.25)	(3.30)	(2.71)	(0.415)			
Polyplastron									**	**	NS
FC	0.87	0.33	0.30	1.23	1.43	1.23	0.90				
	(2.02)	(0.99)	(1.39)	(2.16)	(2.65)	(2.17)	(1.90)	(0.436)			
FM	1.70	NF	0.33	1.20	1.77	2.00	1.17				
	(2.25)	(0)	(0.99)	(1.63)	(1.74)	(2.76)	(1.56)	(0.436)			
FM/L	0.57	NF	0.43	0.53	0.87	0.67	0.51				
	(1.51)	(0)	(0.57)	(1.48)	(1.58)	(1.09)	(1.04)	(0.436)			
CC	3.13	2.53	4.00	3.00	3.23	3.33	3.20				
	(2.93)	(2.78)	(3.49)	(3.43)	(3.45)	(2.94)	(3.17)	(0.436)			
CM	3.33	2.76	0.17	0.23	0.21	0.29	2.51				
	(2.37)	(2.32)	(1.74)	(1.81)	(2.30)	(2.41)	(2.16)	(0.436)			
CM/L[x]	1.72	0.12	NF	NF	0.40	0.92	0.53				
	(1.45)	(0.56)	(0)	(0)	(1.18)	(1.34)	(0.76)	(0.478)			

[b]Other than the values in parentheses, the values shown are means of actual counts (× 10^3 cells / mL). The values in parentheses are the means of $\log_{10}$ transformations of the data.

[b]Transformed ($\log_{10}$) data were used for statistical analyses. ***$P < 0.001$; **$P < 0.01$; *$P < 0.05$; NS = not significant

[d]n = 6 except for CM/L which had had one less animal

[e]NF = not found