ANALYSIS OF PROTOZOAL POPULATION DIFFERENCES IN CATTLE SUBSPECIES

An Undergraduate Research Scholars Thesis

by

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We, Alyson Fontenot[1] and Charlotte Heide[2], certify that all research compliance requirements related to this Undergraduate Research Scholars thesis have been addressed with my Research Faculty Advisor prior to the collection of any data used in this final thesis submission.

This project required approval from the Texas A&M University Research Compliance & Biosafety office.

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ABSTRACT

Analysis of Protozoal Population Differences in Cattle Subspecies

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Previous research suggests that *Bos taurus taurus* cattle (BT) are less feed efficient when fed low-quality forage, produce more methane (CH_4), and exhibit lower levels of N recycling than *Bos taurus indicus* cattle (BI), but the basis of these variations is not well characterized with regards to protozoal digestion of feeds. Differences in feed efficiency among other digestive functions in BT and BI may be closely associated with resident foregut (reticulorumen) protozoa populations, making this area of research valuable for future precision feeding techniques, management of the rumen microbiota, as well as aiding in the reduction of CH_4 production. Samples of rumen fluid from six BT and five BI were analyzed over a period of time concurrent with varying levels of protein supplementation. Classification of two main categories of protozoa, entodiniomorph and holotrich, took place during the process of enumeration. Data for comparisons of BT and BI are presented in the form of protozoa concentration and proportion of holotrich and entodiniomorphs which are used for analyzing hourly data and data compiled for both cattle subspecies. Data collected showed no significant differences between BI and BT for total protozoa concentrations, entodinimorph concentrations, or holotrich concentrations.

Proportional differences between BI and BT on the basis of subspecies was not significant, but differences related to time of collection were significant. Size differences were also observed, but not formally quantified with BI appearing to have larger entodiniomorphs than BT. Further research is planned for a deeper understanding of the protozoal population differences between BI and BT.

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NOMENCLATURE

ADF	Acid Detergent Fiber
BI	*Bos taurus indicus*
BT	*Bos taurus taurus*
DIP	Degradable Intake Protein
GHG	Greenhouse gas
sp.	Species
ssp.	Sub-species
Subf.	Sub-family
UIP	Undegradable Intake Protein
VFA	Volatile Fatty Acid

1. INTRODUCTION

Quantifying differences in digestion, absorption, and metabolism between subspecies of cattle (BT and BI) is a key component in advancing precision feeding and minimizing environmental effects of beef production. Microbial digestion in the reticulorumen of cattle is of primary concern when attempting to analyze differences between BT and BI. Ruminal metabolism differences between the two subspecies can provide insight into dissimilarities in greenhouse gas (GHG) emissions. Bacterial species evaluations have provided some information as to the cause of these differences between BT and BI, but little is known about their protozoal populations and the role or protozoa in digestion. In general, protozoa are classified as small, typically single-celled eukaryotes with a few main ciliated phylogenetic orders of importance in ruminant animals, those being *Entodiniomorphida*, *Prostomatida,* and *Trichostomatida*, the latter two are more commonly referred to by the subclass name “Holotricha" or “holotrich” (Patel and Ambalam, 2018). Characterizing and quantifying the differences in protozoal populations between BT and BI is a task that requires further research to fully understand, and the objective of this experiment is to contribute to the knowledge surrounding ruminal protozoal populations and potential differences between cattle subspecies. In this paper, protozoa will be primarily distinguished at the level of Order or subclass due to the recent debate over the accuracy of species designations; where some researchers believe intraspecies variations are not fully considered (Michatowski, 2005). Accordingly, this study aims to distinguish the differences in composition and concentration of protozoal populations between BT and BI.

1.1 Differences Between BT and BI

The two subspecies of beef cattle, BT and BI, originate from different geographical areas with different climates, resulting in different adaptations. Originating from the domestication of wild aurochs or *Bos primigenius*, BT cattle were first utilized by humans on the European continent at the end of the Neolithic period (Bonfiglio et al., 2012). Breeds that fall under this category are usually classified as "British" or "Continental European", depending on where they resided following their initial domestication. Common examples of British breeds include: Angus, Hereford, and Shorthorn. British breeds are known for their maternal, milking, and marbling ability. Common continental breeds include: Charlois, Chianina, and Limousin. Continental breeds are known for their superior growth and frame size compared to British breeds. Cattle of the BI subspecies are often referred to as Zebu cattle, and they originate from India where they are believed to have descended from the native, wild cattle in India known as *Bos nomadicus* (Naik, 1978). Common breeds of BI cattle include: Nellore, Afrikander, and American Brahman. Phenotypic differences between BT and BI are easily distinguishable. The most notable differences being BI exhibit a large hump located above their shoulder, longer ears, a short hair coat, and excess skin along their neck and underline. Meanwhile, BT exhibit a longer hair coat and lack a hump.

Origination differences have also led to distinct differences in their attributes. Due to their origin in Europe, BT cattle are more adequately adapted for colder, harsher climates. These cattle are the predominant type utilized in the United States' food chain due to their inclination to deposit more marbling than BI, making their beef more palatable and tender (Rodrigues et al., 2017). Higher amounts of marbling observed in BT carcasses ultimately leads to these cattle having greater value in the cattle market, often selling for a higher premium.

Increased muscle mass and tenderness can be related to the increased metabolic rates of BT cattle (Scheffler, 2022). Tenderness results from calpain protease activity in the muscle; increased calpain concentration is related to increased tenderness (Scheffler, 2022). It has been observed that BT have increased calpain concentrations, and this increased proteolytic activity results in increased heat production (Scheffler, 2022). In addition, they are observed to have greater feed intake along with increased weight gain and growth (Scheffler, 2022). Milk production in BT is also considered superior, allowing them to dominate the dairy industry and excel on the maternal side of breeding programs that value complementarity (Syrstad, 1985). It is also known that BT have higher reproductive efficiency than BI due to their younger age of puberty, increased fertility rates, and increased sperm production (Lunstra and Cundiff, 2003). For these reasons, producers in colder climates may opt to utilize BT in their operation.

Originating in tropical and subtropical climates of India has caused BI cattle to have increased heat tolerance and disease resistance. Heat tolerance is one of the best known attributes of BI cattle. They are able to withstand much higher temperatures and levels of humidity due to their superior ability to balance their heat production and heat loss (Utsunomiya et al., 2019). Heat production is related to the metabolic rate of the cattle, with increased metabolic rates resulting in an increase of heat produced (Scheffler, 2022). Size of the organs associated with metabolism and muscle mass can play in metabolic rate (Scheffler, 2022). Zebu cattle have been observed to have smaller livers and hearts; this finding is consistent with BI cattle having decreased metabolic rates (Scheffler, 2022). Metabolic rate can be related to energy maintenance demands. Maintenance demands are lower for BI than their BT counterparts (Frisch and Vercoe, 1977). This finding may alter wide-spread feed formulation practices at the level of breed type, but further accuracy for diet formulation can be achieved if the cause of these differences is

better understood. Increased energy efficiency can be related to decreased fasting heat production which contributes to their lower energy demands (O'Kelley and Spiers, 1992). This coupled with BI's ability to efficiently lose heat to the environment allows BI to more effectively thermoregulate than BT (Scheffler, 2022). Their short hair coat and excess skin also results in increased surface area for heat dissipation (Scheffler, 2022). Disease and insect resistance are also a trademark of BI cattle. Due to centuries of grazing in south-eastern Asia, natural selection has allowed for BI to be resistant to many disease-causing parasites including: ticks, mosquitoes, flies, and helminths (Utsunomiya et al., 2019). Additionally, these cattle are more efficient at digesting poor-quality forages than BT (Scheffler, 2022).

Many common American beef breeds of cattle can be traced back to Zebu origin. A common breed used in American beef production systems is the American Brahman. American Brahman is of the BI subspecies and can be traced back to the Gyr, Guzerat, Nellore, and Krishna Valley breeds of India. In the United States beef production system, BI play a different role than BT. Due to their heat tolerance and disease resistance, BI are primarily located along the Gulf Coast. Compared to BT, they have decreased marbling, tenderness, and flavor, and for this reason, BI influenced cattle are not typically found in production feedlot settings (Scheffler, 2022). A trademark of BI cattle is the heterosis or hybrid vigor that results when they are crossed with BT. Heterosis is an increase in productivity that results from the heterozygosity of alleles between the two subspecies (Arthur et al., 1999). Recent crossbreeding and composite programs have aimed to maximize the beneficial characteristics between the two subspecies, resulting in breeds like Brangus, Simbrah, Beefmaster, and Santa Gertrudis which are popular in tropical and subtropical regions of the United States. Filial one (F1) progeny between BT and BI are favorable for use on the maternal side of commercial cow-calf breeding programs due to their

maternal heterosis. This increase in productivity can be seen as reduced calving intervals, increased lactation length, increased milk yield, and earlier age at puberty, when compared to mid-parent averages (Syyrstad, 1985). Additional heterosis influences include carcass-type trait benefits for pasture-finished cattle on the basis of tenderness and some aspects of marbling (Gama et al., 2013).

1.1.1 Differences in Digestibility and Microbiome

Nutrient digestibility is greater in BI compared to BT when fed low-protein diets, in part due to increased fermentation rates and decreased rumen retention times (Howes, et al., 1963). While many factors contribute to this particular difference in maintenance requirement, microbial populations may play a role in this effect; however, there is limited research on the composition and quantification of their protozoal populations.

Ruminal pH levels are observed to be greater in BI (O'Kelley and Spiers, 1992). Greater ruminal NH_3 levels have also been observed in BI, which has been linked to increased microbiome efficiency (Latham, et al. 2018). Increased NH_3 levels has the potential for BI having an increased rate of microbial digestion compared to BT, which in turn increases microbial protein production (Hunter and Spiers, 2007). Additionally, it has been reported that there is no significant difference in the diversity of the bacterial populations between BT and BI, only a difference in the abundance of specific bacterial species (Latham, et al. 2018). These differences are less noticeable when cattle are fed adequate amounts of N (Hunter and Siebert, 2007). Past research has stated that BI have higher protozoal concentrations, specifically protozoa in the order of *Entodiniomorphida* and the holotrich genus *dasytricha*, compared to BT (O'Kelley and Spiers, 1992). It can be presumed that increased levels of NH_3 in BI rumens benefits microbial protein synthesis, providing a food source for predatory entodiniomorphs. This concentration

difference may play a role in determining the differences in fermentation and nutrient absorption between BI and BT. Varying concentrations of protozoa in the rumen between the two cattle subspecies results in different end products for digestion; therefore, resulting in differences in feed efficiency (O'Kelley and Spiers, 1992).

1.2 Role of Protozoa in the Rumen

Protozoa comprise from 0 to 50% of the microbial mass in the rumen (Belanche et al., 2011). Protozoa are outnumbered by bacteria in the rumen; however, they are much larger in size (protozoa: 10–200 μm, bacteria: 0.5–2 μm; Williams and Coleman, 1992). Protozoa are not essential for the ruminant survival; however, they are important for foregut microbial digestion including, but not limited to: fiber digestion, methanogenesis, and microbial protein breakdown. It is estimated that 50-70% of ruminal fibrolytic activity is attributable to ciliate protozoa (Michatowski, 2005). Presence of specific ciliate protozoa increases the population of fiber-degrading bacteria from 15.48 $\times 10^8$/g rumen content to 78.540 $\times 10^8$/g rumen content; Williams and Withers, 1991). Protozoa not only degrade polysaccharides, but they also create a more favorable pH for cellulolytic bacteria (Li et al., 2018). This occurrence can be explained by the activity of specific protozoa that compete with lactic-acid producing bacteria for non-structural carbohydrates and produce a less-acidic product (Michatowski, 2015). During fermentation of structural and non-structural carbohydrates, the protozoa produce hydrogen (H_2) and volatile fatty acids (VFA), production of which serves as an energy source for the ruminant, specifically the VFA acetate (Li et al., 2018). In fact, VFA provide up to 70% of the energy required by the animal (Li et al., 2018).

H_2 production occurs in the hydrogenosome of specific ciliate protozoa (Tymensen, et al. 2012). H_2 produced by protozoa as well as carbon dioxide (CO_2) found in the rumen, allows for a

symbiotic relationship with methanogenic bacteria and archaea, and this relationship is responsible for 37% of total CH_4 produced in the rumen (Finley, et al. 1994). The extent of interspecies hydrogen transfer between protozoa and methanogens is demonstrated by complete ruminal defaunation, which led to an 11% decrease in methane production by cattle (Newbold et al., 2015). Globally, methanogenesis is of concern because of methane's role as a GHG. Animal performance and feed efficiency are reduced by the energy losses associated with methane production. Methane emissions from the ruminant accounts for 2-12% of energy losses (Li et al., 2018).

Microbial protein synthesis and degradation is vital to meeting the animal's metabolizable protein requirement. Research by Koeing et al, reported 40-90% of protein reaching the duodenum is microbial protein (2000). Protozoa lack the ability to synthesize amino acids from ammonia (NH_3); therefore, they rely on predation of bacteria as a source of free amino acids and peptides (Jouany and Ushida, 1999). Predation results in intraruminal recycling of nitrogen (N), primarily through the engulfment of bacteria by the protozoa (Koeing et al., 2000). Protozoal degradation of bacterial protein results in increased ruminal NH_3 concentrations (Wallace et al., 1987). Complete ruminal defaunation decreased microbial N recycling and increased bacterial-N duodenal flow; however, efficiency of microbial protein synthesis was not consistent (Belanche et al., 2011). Bacteria are the primary contributor to microbial protein synthesis, and complete ruminal defaunation results in an increase of N flow by 60% (Koeing et al., 2000). However, complete ruminal defaunation did not affect overall N recycling (Koeing et al., 2000).

Protozoa concentrations are affected by the feeding pattern of the animals. O'Kelley and Spiers discovered a decrease in protozoal numbers 4-8 hours post-feeding, with the

concentrations increasing throughout the day (1992). Protozoa have a slower passage rate than ruminal liquid passage rate, as some protozoa adsorb to feed particles or sequester in the rumen epithelium (O'Kelley and Spiers, 1992).

1.3 Holotrichs (Trichostomatida and Prostomatida) vs. Entodiniomorphs

Protozoa from the orders Trichostomatida and Prostomatida, usually referred to as holotrichs, and protozoa from the order Entodinipomorphida, referred to as entodiniomorphs, are the two main groups of ciliate protozoa occupying the rumen. Animals faunated with solely holotorich protozoa indicated that holotorich play a greater role in methanogenic activity via interspecies hydrogen transfer quantified by an approximated 54% increase in methanogenesis when compared to defaunted animals (Belanche et al., 2012). Increased activity of their hydrogenosome increases methane production within cattle containing a substantial population of holotrichs (Newbold et al., 2015). Additionally, holotrichs do not consume fibrous material but instead ingest soluble starches and other non-structural carbohydrates, which is beneficial to cattle fed high concentrate diets as they may outcompete bacteria associated with acidosis (Michatowski, 2005). Holotrichs play a much smaller role in nutrient digestion compared to entodiniomorphs when cattle are fed high protein diets with the most commonly found entodiniomorph species, *Entodinium,* responsible for approximately 70-75% of protozoal bacterial predation and the two most common holotrich species, *Dasytricha* and *Isotricha*, contributing averages of 0.6 to 1.2% and 0.2 to 0.5% of protozoan bacterial predation, respectively (Belanche et al., 2012).

Entodiniomorphs have a greater affinity for rumen bacteria, peptides, and structural carbohydrates (Newbold et al., 2015). They play a more important role in ruminant livestock nutrition through their fibrolytic activity and by predating bacteria, which decreases microbial

protein available to the animal and increases levels of ruminal NH_3 concentration approximately 26% when compared to defaunated animals (Newbold et al., 2015). Feed protein also escapes degradation and is more available to the animal in the absence of entodiniomorphs which contributes to increased feed efficiency (Newbold et al., 2015). Environmentally, this is of importance because of the implications of increased nitrogen excretion both as a loss of feed efficiency in the cattle, leading to inefficient use of resources, as well as the potential to contribute to eutrophication of surrounding water sources.

1.3.1 Protozoa Morphology and Activity

Protozoa considered holotrich (Trichostomatida order) are primarily of the classification *Dasytricha* sp. and *Isotricha* spp., although other species may reside in the rumen microbiome. Entodiniomorph (Entodiniomorphida order) protozoa in the rumen are typically of the *Epidinium* sp., *Entodinium* sp., as well as the Diplodiniinae subf., and Orphryoscolex subf. classifications.

Morphological descriptions provide a baseline understanding of the physiology, activity, and phylogenetic relationships among protozoa. The morphology of *Dasytricha* consists of oval-shaped cells that have full ciliature, spiral longitudinal cilia, central macro- and micronuclei, no contractile vacuoles, and a flexible pellicle (Purevtsogt et al., 2016). This genus has notable glucosidase and cellobiosidase activity and consumes mostly nonstructural and soluble carbohydrates (Michatowski, 2015). The other holotrich classification of concern are the subspecies in the genus *Isotricha,* which have full ciliature, a flexible pellicle, kidney-shaped macronucleus, absent skeletal plate, and an absent contractile vacuole (Purevtsogt et al., 2016). Similar to the other holotrich protozoa, it utilizes nonstructural and soluble carbohydrates as its primary source of food.

Within the Entodiniomorphida order, it is common to have adoral ciliature and a rigid pellicle, but some morphological characteristics differ among this classification of microbes (Purevtgost et al., 2016). In addition to the commonalities in Entodiniomorphida organisms, *Epidinium* has an ovoid macronucleus, 3-sectioned skeletal plate, and an unbranched and thornless tail (Purevtgost et al., 2016). Similar to *Epidinium,* the subfamily Ophryoscolex displays a 3-sectioned skeletal plate and an ovoid macronucleus; however, they have tail thorns and plentiful contractile vacuoles (Purevtgost et al., 2016). Diplodiniinae have the characteristics associated with the Entodiomorphida order as well as an ovoid or crook-shape macronucleus (Purectgost et al., 2016). Unlike other entodiniomorphs and similar to holotrichs, *Entodinium* lacks a skeletal plate, but still shares the ovoid macronucleus (Purevtgost et al., 2016).

Preferred substrates and activity within the Entodiniomorphida order revolve around particulate matter, starch, and structural carbohydrates (Michatowaski, 2015). More is known about the enzymatic activity of *Epidinium* which is shown to have Beta-endoglucanases as well as Beta-endoxylanases (Michatowski, 2015).

1.4 Industry Impact

Nutrition related costs are arguably the largest or second largest for producers, but precision feeding reduces the oversupplying of nutrients reducing the cost to producers. Understanding the role of protozoa in BT and BI digestion can assist in the formulation of more specific diets that maximize or consider protozoal impacts on digestion. Precision feeding can ultimately lead to the decrease of nutrient excrement from animals, both benefiting the producer and the environment. Additionally, a more comprehensive understanding of methanogen and protozoa symbiosis can lead to decreased methane emissions from cattle.

1.5 Discovery of Methods

Similar research has been conducted by Belenche et al, where protozoal populations of sheep were collected for enumeration and DNA analysis (2015). Samples of rumen fluid were collected and combined with formalin for preservation (Belenche et al., 2015). Formalin and formaldehyde-based preservatives for fixing and inactivating microorganisms greatly outperform other methods of preservation (Lee et al., 1992). A phosphate-methyl-green and glycerol buffer used for storage and visualization of the protozoal samples was prepared using information from methods detailed by Boyne et al. (1957) and Dehority (1984), who also advanced the method of enumeration by using a disposable cell counter. Rumen fluid collection technique occurred using a suction-strainer placed below the fiber mat within the rumen, at the advice of ruminant nutritionist Dr. Tryon Wickersham, instead of capturing solid rumen contents and utilizing cheesecloth as a straining method. No studies have confirmed the presence of differences in rumen protozoa concentration within the fluid versus fiber mat and none have addressed possible issues with deviating from the standard use of cheesecloth to collect rumen microbiota samples.

2. METHODS

Five BI (Brahman) steers and six BT (Angus) steers of approximately the same age were kept in climate-controlled barn that contained metabolism crates and stalls for the trial. Prior to starting the project, the steers were housed in a small paddock with access to a barn. Steers were randomly assigned to one of two groups to facilitate sample collection and work within the restrictions of the research facilities. Steers were fitted with ruminal cannulas for sampling digesta. A 6×6 Latin square experiment was used to assign treatment to the steers. The steers were adapted to treatments for 7-d and then moved to the metabolism crates on d 8. Cattle were allowed ad libitum access to water and a mineral block with low-quality hay provided at 130% of the expected dry matter intake. Supplements of varying amounts degradable intake protein (DIP) and undegradable intake protein (UIP) were assigned within the Latin square. A diet of only low-quality forage was used as the control diet. The five diets are as follows:

1. Control: 0 mg CP/ kg BW (CON)
2. Low intake DIP: 70% DIP and 30% UIP at 350 Mg CP/kg (LD)
3. Low Intake UIP: 50% DIP and 50% UIP at 350 Mg CP/kg (LU)
4. High Intake DIP: 70% DIP and 30% UIP at 700 Mg CP/kg (HD)
5. High Intake UIP: 70% UIP at 700 Mg CP/ kg (HU)

All supplements, excluding the control, consisted of 43% CP. Rumen fluid samples were collected on d 14 of each period just prior to feed (h 0) and 4 h after feeding. Prior to freezing, rumen fluid samples were combined with formalin in a 1:1 fashion and further diluted with a glycerol-methyl green buffer at a 1:5 dilution with a final dilution of 1:10. Protozoal enumeration of the pre-prepared and thawed samples occurred using a disposable cell counter (Watson Fuchs-

Rosenthal) with 12uL of the dilute sample to estimate the concentration of the protists, microscopically. During enumeration, identification of protozoa as either entodiniomorphs or holotrichs was conducted to quantify the most common types of protozoa found in each cattle subspecies. Statistical analyses using the SAS program were done to determine if the differences between protozoal populations in the subspecies were significant.

3. RESULTS

As seen in *Table 3.1*, concentration of protozoa in BI at hour zero yielded a value of 57,290 cells per mL rumen fluid and 57,240 cells per mL at hour four, while BT averaged 53,140 cells per mL rumen fluid at hour zero and 52,730 cells per mL rumen fluid at hour four both with a standard error (SE) of 5880 cells per mL rumen fluid. P-values for subspecies, time, and subspecies by time are 0.41, 0.96, 0.97, respectively, indicating there was not a significant effect.

Entodiniomorph concentration for BI at hour zero was 44,930 cells per mL rumen fluid and 48,550 cells per mL rumen fluid at hour four. Entodiniomorph concentration in BT at hour zero was 40,020 cells per mL rumen fluid and 41,150 cells per mL rumen fluid at hour four. The SE for entodiniomorph concentration was 5,030 cells per mL rumen fluid, and the P-values were 0.18, 0.54, and 0.75 for subspecies, time, and subspecies by time, respectively, indicating there was not a significant effect.

For BI at hours zero and four, there were concentrations of 12,350 and 8,488 cells per mL rumen fluid compared to BT with values of 13,100 cells per mL rumen fluid for hour zero and 11,730 cells per mL rumen fluid at hour four. The SE was 2,060 cells per mL rumen fluid and resulting in a p-value of 0.31 for subspecies effect, 0.09 for time effect, and 0.40 for the subspecies by time interaction.

Entodiniomorph proportion at hour zero was 75.19% of the total BT protozoal population and 78.58% of total BI protozoal population. At hour four, BT entodiniomorphs were 77.49% of the total protozoal population and 84.40% of the BI protozoal population. P-values for subspecies effect of 0.11, 0.08 for the time effect, and 0.42 for subspecies by time

interaction. This data indicates a tendency for time to have an effect on the proportion of entodiniomorphs.

At hour zero, holotrichs made up 21.42% of total protozoal population in BI and 24.73% of total protozoal population in BT. Holotrich proportion at hour four for BI was 15.6% of the total protozoal population and 23.14% of BT's total protozoal population. These results had a SE of 3.125% with p-values for the subspecies effect, time effect, and subspecies by time interaction of 0.09, 0.09, and 0.3269. This data indicates a tendencies for time and subspecies to have an effect on the proportion of holotrichs.

Table 3.1: Ruminal Protozoa Concentration and Proportions

	Time			p-value		
	Hr 0	**Hr 4**	**SEM**	**Subspecies**	**Time**	**S×T**
Total Protozoa						
BI	57290	57240	5884	0.41	0.96	0.97
BT	53140	52730				
Entodiniomorph						
BI	44930	48550	5030	0.18	0.54	0.75
BT	40020	41150				
Holotrich						
BI	12350	8480	2057	0.31	0.09	0.41
BT	13100	11730				
Entodiniomorph Proportion						
BI	78.58%	84.40%	4.00%	0.11	0.07	0.42
BT	75.19%	77.49%				
Holotrich Proportion						
BI	21.42%	15.6%	3.13%	0.09	0.09	0.33
BT	24.73%	23.14%				

Note: Concentration values reported as cells per mL rumen fluid

3.1 Discussion of Results

3.1.1 Concentration Differences

Ruminal concentration results showed no significance for total protozoal concentration differences between subspecies (p-value > 0.1) or time of sample (p-value > 0.1). These findings are inconsistent with that of O'Kelley and Spiers who found a significant increase in total protozoa populations in BI compared to BT (1992). There was no significant difference between the entodiniomorph concentration between BT and BI (p-value > 0.1) or between hour zero and four (p-value > 0.1). Holotrich concentration differences were not significant between subspecies (p-value > 0.1) but were significantly different between hour zero and four (p-value < 0.1) with hour zero having a greater concentration.

Entodiniomorph and holotrich concentrations are inconsistent with O'Kelley and Spiers who found greater concentrations of entodiniomorph in BI and greater concentrations of holotrich in BT (1992). A decrease in holotrich concentration at hour four is consistent with O'Kelley and Spiers' findings (1992). This concentration difference is likely attributed to an increase in dilution in the rumen with increased consumption of feed and water. The lack of significant differences could be due to the varying amounts of protein supplemented in the treatments as well as the size of the experimental population. Varying amounts of protein supplementation could potentially affect the rumen microbiome, specifically entodiniomorph populations due to their predatory nature with rumen bacteria. Further research is needed to determine the significance of protozoal concentration differences related to levels of protein supplementation between BI and BT and the addition of more observations should be included to improve significance.

Increased ruminal protozoa concentration is likely associated with the idea of BI cattle exhibiting increased feed efficiency. More specifically, greater entodiniomorph concentration can result in an increased rate of breakdown of low-quality forage from their fibrolytic activity, which aids in explaining the observation that BI are more feed efficient. Increased feed efficiency assisted by superior N recycling exhibited by BI likely benefits protozoal populations by providing one of their food sources, bacteria, with N for protein synthesis and growth. Entodiniomorphs are more closely associated with bacterial predation, so it was expected that this classification of protozoa would be greater in BI than BT. This expectation was not supported as it was experimentally determined that protozoal concentrations were not significantly different between cattle subspecies. Protozoal concentration has been reported to be 10^5-10^6 cells per mL of rumen fluid (Li et al., 2018). The present study reported average concentrations of 10^4 cells per mL of rumen fluid for both BI and BT, an observation that can be caused by the low-quality forage diet fed to the steers. Minimal protein supplementation could also cause decreased numbers of ruminal protozoa.

A non-documented observation found that entodiniomorphs in BI were larger in size compared to those found in BT. Size differences among protozoa may have been rooted in phylogenetic differences among the types of entodiniomorphs observed. Based upon pictures provided in a study by Purevtsogt et al., the larger sized protozoa found more abundantly in BI rumen samples appeared to be of the *Diplodinium* and Ophryoscolex classifications (2016). Entodiniomorphs that made up the majority of BT rumen samples appeared to be of *Epindinium* origin, based upon pictures and morphological descriptions provided by Purevtsogt et al. (2016). *Diplodinium* and Ophryoscolex conduct notable amounts of cellulose digestion that can contribute to 50-70% of fibrolytic activity in the rumen and may provide insight into the

increased digestibility of forages in BI (Michatowski, 2015). In a study on small ruminants, Michatowski reported that 50% of VFA were a product of ciliates, with further analysis of the protozoal populations yielding majority Ophryoscolex protozoa (2015). Larger *Epidinium* also contribute to cellulose digestion but are the type of entodiniomorph most closely associated with methanogenic activity (Kišidayová et al., 2000). These results may align with possible digestibility and energetic differences between the cattle subspecies. While these observations were not measured, this discovery could lead to further research regarding protozoa size and further phylogenetic differences between subspecies rather than concentration differences.

3.1.2 Proportional Differences

Subspecies differences in proportion of entodiniomorphs were found to be not significant (p-value > 0.05). However, non-subspecies specific, hourly differences, though not significant, showed a trend towards significance, with a general increase in entodiniomorph proportion from hour one to hour four (p-value < 0.1). Holotrich proportional differences among subspecies also had insignificant values, but did show a statistical tendency (p-value < 0.1) with BT typically having higher proportions of holotrich protozoa. Non-subspecies related time differences also showed a statistical tendency, with a decrease in proportion of holotrich protozoa from hour zero to hour four (p-value < 0.10).

Subspecies differences in holotrich and entodiniomorph concentrations that occurred could arise from differences in the rumen temperature, pH, or bacterial populations in BT versus BI. It was expected that because bacterial populations tend to be greater in BI, this could lead to increased predatory behavior from protozoa in the rumen, specifically affecting the proportion of entodiniomorphs (O'Kelley and Spiers, 1992). Though this was not experimentally determined, the use of a larger population of animals and suppression of confounding variables such as diet

differences, may have led to different results. If this is true, future research should be conducted to better understand the proposed phenomena. Hourly differences observed could be a result of the type of substrate left in the rumen. For instance, higher levels of fiber available may be conducive to an increased proportion of entodiniomorphs, because of their higher cellulolytic activity than holotrichs (Newbold et al., 2015).

4. CONCLUSION

Ruminal protozoa populations account for nearly half of ruminal biomass and can be responsible for a significant amount of digestion in the ruminant. The two major types, entodiniomorphs and holotrich, consume different feedstuffs and contribute to different end products of digestion. When considering cattle consuming low-quality hay, entodiniomorphs are the major contributor to digestion of the fiber, and the predation of ruminal bacteria. This predation of bacteria in the rumen leads to increased ruminal N concentration which is interconnected with N recycling within the animal. Previous research has indicated that BI have higher total concentrations of protozoa, with increased proportions of entodiniomorphs. However, this observation was not supported by the results of the present study. Differences in holotrich and entodiniomorph proportions based on hour of collection were reported. It has been determined that BI are more feed efficient with increased N recycling compared to BT. Further research is needed to determine whether this difference is related to protozoal concentration, proportions, or size. A deeper knowledge of the topic could lead to increased sustainability of the beef industry through precision feeding techniques and control of protozoal populations.

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