

Ruminal Degradation of Polyhydroxyalkanoate and Poly(butylene succinate-*co*-adipate)

Hailey Galyon

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Rebecca Cockrum, Chair
Gonzalo Ferreira
Abby Whittington

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ABSTRACT

The occurrence of plastic impaction in ruminants is a growing concern. As indiscriminate feeders, cattle may consume plastic foreign materials incorporated into their diets and it is currently estimated that 20% of cattle contain plastic foreign materials in their rumen. These materials are indigestible and accumulate for the lifetime of the animal. As these materials accumulate, they may reduce feed efficiency and production by erosion and ulceration of rumen epithelium, stunting of papillae, blockage of the reticulo-omasal orifice, and leaching of toxic heavy metals. It is necessary to reduce the incidences of plastic impaction in domestic ruminants. Using polyhydroxyalkanoate (PHA) and poly(butylene succinate-*co*-adipate) (PBSA) biodegradable materials for feed storage products such as bale netting could reduce the incidences and effects of polyethylene-based plastic impaction in ruminants. The objectives of these studies were to evaluate the degradability of PHA and PBSA materials in the reticulorumen via in vitro, in situ, and in vivo methods. Our hypothesis was that these materials would degrade in the rumen and that a melt-blend of PHA and PBSA may degrade faster than its individual components.

An in vitro study incubated a proprietary PHA-based polymer, PBSA, and PBSA:PHA melt blend nurdles, and forage controls in rumen fluid for up to 240h in Daisy^{II} Incubators. Mass loss was measured, and digestion kinetic parameters were estimated. Thermogravimetric and differential scanning calorimetry analyses were conducted on incubated samples. Results indicated that the first stage of degradation

occurs within 24h and PHA degrades slowly. Degradation kinetics demonstrated that polymer treatments were still in the exponential degradation phase at 240h with a maximum disappearance rate of 0.0031%/h, and mass loss was less than 2% for all polymers. Melting temperature increased and onset thermal degradation temperature decreased with incubation time, indicating structural changes to the polymers starting at 24h.

Further in situ degradation, however, indicated these biodegradable materials degrade at more accelerated rates in the rumen. Polyhydroxyalkanoate, PBSA, PBSA:PHA blend, and low-density polyethylene (LDPE) films were incubated in the rumens of three cannulated, non-lactating Holsteins for 0, 1, 14, 30, 60, 90, 120, and 150d. In situ disappearance (ISD) and residue length were assessed after every incubation time. Polyhydroxyalkanoate achieved 100% degradation by 30d, with initiation occurring at 14d indicated by ISD and a reduction in residue length. The fractional rate of disappearance of PHA was 7.84%/d. Poly(butylene succinate-*co*-adipate) and Blend did not achieve any significant ISD, yet fragmentation of PBSA occurred at 60d and the blend at just 1d likely due to abiotic hydrolysis. Low-density polyethylene achieved no ISD and residue length did not change over incubation time. From these results, we proposed a PBSA:PHA blend is a valid alternative to polyethylene single-use agricultural plastic products based on its fragmentation within 1d of incubation.

Administration of PBSA:PHA film boluses compared to LDPE films and a control further supported this dissemination. Holstein bull calves (n = 12, 62 ± 9d, 74.9 ± 8.0kg) were randomly allocated to one of three daily bolus treatments: 13.6g of PBSA:PHA in 4 gelatin capsules (Blend), 13.6g of LDPE in 4 gelatin capsules (LDPE),

or 4 empty gelatin capsules (Control) for 30d. Hemograms were conducted on blood samples collected on d0 and d30. On d31, animals were sacrificed to evaluate gross rumen measurements and pathology, determine papillae length, and characterize polymer residues present in rumen contents. Feed intake, body weight, body temperature, and general health were determined throughout the study. No animals presented any symptoms related to plastic impaction and animal health was not particularly affected by treatment. Daily grain and hay intake, body weight, rectal temperature, hematological parameters, gross rumen measurements and pathology, and rumen pH and temperature were not affected by treatment. There was evidence that degradation of PBSA:PHA may release byproducts that support rumen functionality. Methylene blue reduction time of Blend calves tended to be decreased by 30% compared to LDPE calves, and caudal ventral papillae length of Blend calves was 50% longer than those of Control animals. Though studies are needed to specifically elucidate the production of byproducts due to degradation of PBSA:PHA and their correlations. Polymer accumulation and residue length differed among treatments. Calves dosed with LDPE retained 6.7% of the dosed polymer, undegraded, while Blend calves retained 0.4% of the dosed polymer. The polymer residues in Blend calves were 10% of their original size.

Single-use agricultural plastics developed from PBSA:PHA may be a suitable alternative to LDPE-based products in the case of ingestion in ruminants due to no acute health inflictions, fragmentation of polymers with 1d, and improved clearance from the reticulorumen. As such, utilization of these materials may reduce the incidences of plastic impaction in ruminants in commercial operations. Further long-term feeding studies are needed to evaluate specific byproduct production of PBSA:PHA and their potential

influences on rumen function and animal health and production in normal commercial conditions.

Ruminal Degradation of Polyhydroxyalkanoate and Poly(butylene succinate-*co*-adipate)

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GENERAL AUDIENCE ABSTRACT

Plastic feed-storage materials may unintentionally be incorporated into animal feeds. Net wraps and bale twines may be stuck or left on forages when they are ground and incorporated into mixed rations. As cattle are largely non-selective, they may inadvertently consume these plastic materials. Approximately 20% of cattle contain plastic foreign materials in their rumen. These materials are indigestible and accumulate for the animal's lifetime. As plastics build up in the rumen, they may reduce feed efficiency, body weight, and milk production by damaging the rumen lining, blocking the digestive tract, and leaching toxic heavy metals. Therefore, it is necessary to reduce the incidences of plastic impaction in domestic ruminants to improve their health and productivity. Using biodegradable materials that are degraded by bacteria, such as polyhydroxyalkanoate (PHA) and poly(butylene succinate-*co*-adipate) (PBSA), for feed storage products could reduce the occurrence and effects of plastic impaction in ruminants due to the materials' potential degradation in and passage from the rumen. The objectives of these studies were to evaluate the breakdown of PHA and PBSA materials in the rumen. Our hypothesis was that these biodegradable materials would degrade in the rumen and that a blend of PHA and PBSA may degrade faster than its individual components.

In our first study, PHA, PBSA, a PBSA:PHA blend, and forage controls were incubated in rumen fluid for up to 240h. Mass loss, degradation rate, and the structure of polymers were determined over incubation time. Results indicated that biodegradable

polymers may begin to break down within 24h. Polymer treatments were still in the early stages of degradation at 240h with a maximum degradation rate of 0.0031%/h, and mass loss of polymers was less than 2%. However, within 24h, the structures of polymers may have altered to promote future degradation at longer incubation times.

Accelerated degradation was observed when PHA, PBSA, PBSA:PHA (Blend), and polyethylene (LDPE) films were incubated in the rumens of three Holstein cows up to 150d. Mass loss and the length of the remaining polymers were assessed monthly. Polyhydroxyalkanoate began to degrade by 14d and completely degraded by 30d with a disappearance rate of 7.84%/d. The remaining polymer did not achieve any mass loss. However, PBSA and Blend residue size began to decrease by 60d and 1d, respectively. Based on Blend's structural degradation within 1d of incubation that may promote its clearance from the rumen if ingested, we proposed that the material may be an alternative to polyethylene single-use agricultural plastic products.

When Blend films were fed to calves, breakdown of the material further supported our dissemination that PBSA:PHA may be a suitable alternative to LDPE in the case of animal ingestion. Holstein bull calves ($n = 12$, 62 ± 9 d, 74.9 ± 8.0 kg) were randomly allotted to one of three daily bolus treatments: 13.6g of PBSA:PHA (Blend), 13.6g of polyethylene (LDPE), or no polymer (Control) distributed over 4 gelatin capsules for 30d. Feed intake, body weight, body temperature, and general health were determined throughout the study. Blood analyses were conducted on blood samples collected before and after the experimental period. On d31, animals were sacrificed to evaluate rumen growth and health, measure rumen papillae length, and describe polymers that may reside in the rumen. No animals presented any signs related to plastic impaction

and animal health was not particularly affected by treatment. Daily grain and hay intake, body weight, rectal temperature, blood parameters, and rumen growth and health were not affected by treatment. There was evidence that degradation of Blend may support rumen function. Methylene blue reduction time of Blend calves tended to be decreased by 30% compared to LDPE calves, which indicates the rumen microbiome of Blend calves may better ferment feeds. Papillae length of Blend calves were also 50% longer than those of Control animals, which would improve the absorption of nutrients. Byproduct formation from Blend degradation could explain this; however, studies are needed to specifically elucidate the production of byproducts and their relationship to rumen function. Polymer accumulation and residue length differed among treatments. Calves dosed with LDPE retained 6.7% of the dosed polymer, undegraded, while Blend calves retained 0.4% of the dosed polymer. The polymer residues in Blend calves were 10% of their original size.

Single-use agricultural plastics developed from PBSA:PHA may be a suitable alternative to polyethylene-based products in the case of ingestion in ruminants due to no short-term health inflictions, the reduced polymer size within 1d, and improved clearance from the rumen. As such, utilization of these materials may reduce the incidences of plastic impaction in ruminants in commercial operations. Further long-term feeding studies are needed to evaluate specific byproduct production of PBSA:PHA and their potential influences on rumen function and animal health and production in normal commercial conditions.

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TABLE OF CONTENTS

Abstract	ii
General Audience Abstract	vi
Acknowledgments	ix
Table of Contents	x
List of Abbreviations	xiii
List of Tables	xv
List of Figures	xvi
Introduction	1
Chapter 1: LITERATURE REVIEW	3
1. Agricultural Plastics and ruminant health	3
2. Polyhydroxyalkanoate	6
2.1 <i>Commercial production</i>	7
2.2 <i>Microbial degradation</i>	8
2.3 <i>Abiotic Degradation</i>	10
2.4 <i>Applications</i>	10
3. Ruminal Fermentation	12
3.1 <i>Rumination</i>	12
3.2 <i>Fermentation</i>	13
3.3 <i>Fate of Fermentative End Products</i>	15
3.4 <i>Maintenance of the Rumen Environment</i>	15
4. Potential Ruminant Degradation of Biodegradable Polymers	17
4.1 <i>Rumen Ecology</i>	18
4.2 <i>Rate of Degradation</i>	19
4.3 <i>Effect of Degradation Byproducts</i>	20
4.4 <i>Potential Histopathological Effects</i>	22
5. Conclusions	23
6. Tables	25
7. References	26

Chapter 2: DIGESTIBILITY KINETICS OF POLYHYRDOXYALKANOATE AND POLY(BUTYLENE SUCCINATE-CO-ADIPATE) AFTER IN VITRO FERMENTATION IN RUMEN FLUID	32
1. Abstract	32
2. Introduction	33
3. Materials and Methods	37
3.1 <i>Animal Care and Use</i>	37
3.2 <i>Sample Preparation for In Vitro Disappearance</i>	37
3.3 <i>In Vitro Disappearance</i>	38
3.4 <i>Thermochemical Analysis</i>	40
3.5 <i>Statistical Analysis</i>	41
4. Results	42
4.1 <i>In Vitro Disappearance of Biodegradable Polymers</i>	42
4.2 <i>Degradation Kinetics</i>	42
4.3 <i>Thermochemical Analysis</i>	43
5. Discussion	44
6. Conclusion	50
7. Tables	52
8. Figures	53
9. References	57

Chapter 3: LONG-TERM IN SITU RUMINAL DEGRADATION OF POLYHYDROXYALKANOATE, POLY(BUTYLENE SUCCINATE-CO-ADIPATE), AND LOW-DENSITY POLYETHYLENE IN HOLSTEIN DAIRY CATTLE	63
1. Abstract	63
2. Introduction	64
3. Materials and Methods	67
3.1 <i>Animal Care and Use</i>	67
3.2 <i>Sample Preparation for In Situ Disappearance</i>	67
3.3 <i>In Situ Disappearance</i>	68
3.4 <i>Statistical Analysis</i>	69
4. Results	69
4.1 <i>In Situ Disappearance of Polymers</i>	69
4.2 <i>Polymer Residue Length</i>	70
5. Discussion	71
6. Conclusion	75
7. Figures	77
8. References	81

Chapter 4: CLEARANCE OF POLY(BUTYLENE SUCCINATE-CO-ADIPATE):POLYHYDROXYALKANOATE MELT-BLEND AND LOW-

DENSITY POLYETHYLENE FILMS FROM THE RUMENS OF HOLSTEIN BULL CALVES	86
1. Abstract	86
2. Introduction	87
3. Materials and Methods	89
3.1 <i>Treatment Preparation</i>	89
3.2 <i>Calf Health and Management</i>	90
3.3 <i>Hematological Evaluation</i>	92
3.4 <i>Tissue Collection and Gross Rumen Measurements</i>	92
3.5 <i>Rumen Histological Measurement</i>	93
3.6 <i>Polymer Residues</i>	94
3.5 <i>Statistical Analysis</i>	94
4. Results	95
4.1 <i>Animal Growth and Health</i>	95
4.2 <i>Hematological Parameters</i>	96
4.3 <i>Rumen Measurements</i>	97
4.4 <i>Papillae Measurements</i>	97
4.5 <i>Polymer Residues</i>	98
5. Discussion	99
6. Conclusion	108
7. Tables	110
8. Figures	114
9. References	118
 Chapter 5: CONCLUSION AND IMPLICATIONS	122
1. Summary and Future Studies	122

LIST OF ABBREVIATIONS

CaD = caudal dorsal

CaV = caudal ventral

CrD = cranial dorsal

CrV = cranial ventral

DSC = differential scanning calorimetry

EDTA = ethylenediaminetetraacetic acid

ESR = erythrocyte sedimentation rate

Hb = hemoglobin

ISD = in situ disappearance

LDPE = low-density polyethylene

LSM = least squared mean

MBRT = methylene blue reduction time

MCHC = mean corpuscular hemoglobin concentration

MCV = mean corpuscular volume

MPV = mean platelet volume

PBSA = poly(butylene succinate-*co*-adipate)

PCL = polycaprolactone

PHA = polyhydroxyalkanoate

PHB = poly(hydroxybutyrate)

PHBV = poly(hydroxybutyrate-*co*-valerate)

PHV = poly(hydroxyvalerate)

RBC = red blood cell

RDW = red cell distribution width

SEM = standard error of the mean

T_d = onset thermal degradation temperature

TGA = thermogravimetric analyses

T_m = melting temperature

VFA = volatile fatty acid

VITALS = Virginia Tech Animal Laboratory Services

WBC = white blood cell

LIST OF TABLES

<u>Table 1.1.</u> Short-chain and medium-chain length polyhydroxyalkanoates, as adapted by Tan et al. (2014).	25
<u>Table 2.1.</u> Ruminal disappearance kinetics parameters of alfalfa hay and corn silage forage controls and proprietary polyhydroxyalkanoate-based polymer nurdle (PHA), poly(butylene succinate-co-adipate) nurdle (PBSA), PBSA:PHA melt blend nurdle (90% wt PBSA, 10% wt PHA) (Blend), and a PBSA:PHA melt blend (90% wt PBSA, 10% wt PHA) filament (Filament).....	52
<u>Table 4.1.</u> Hematological parameters of Holstein bull calves at 0d (Initial) and 30d (Final) of administration of either 4 empty gelatin capsules (Control), 13.6g of a Poly(butylene succinate-co-adipate) and Polyhydroxyalkanoate melt-blend in 4 gelatin capsules (Blend), or 13.6g of Low-density Polyethylene in 4 gelatin capsules (LDPE).. ..	110
<u>Table 4.2.</u> Rumen parameters of Holstein bull calves after 30d of administration of either Control, Blend, or LDPE treatment	111
<u>Table 4.3.</u> Frequency (%) of gross pathology scores of rumen epithelium in the cranial dorsal (CrD), caudal dorsal (CaD), cranial ventral (CrV), and caudal ventral (CaV) regions of Holstein bull calves after 30d of administration of either Control, Blend, or LDPE treatment	112
<u>Table 4.4.</u> Frequency (%) of rumen content scores of Holstein bull calves after 30d of administration of either Control, Blend, or LDPE treatment.....	113

LIST OF FIGURES

Figure 2.1. In vitro disappearance (%) of proprietary polyhydroxyalkanoate-based polymer nurdle (PHA), poly(butylene succinate-co-adipate) nurdle (PBSA), PBSA:PHA melt blend nurdle (90% wt PBSA, 10% wt PHA) (Blend), and a PBSA:PHA melt blend (90% wt PBSA, 10% wt PHA) filament (Filament) fermented in rumen fluid for up to 240h. When comparing nurdles, bag type and all its interactions, as well as the interaction of treatment and time, were not significant. Treatment: $P = 0.10$. Time: $P < 0.0001$. When comparing Blend and Filament treatments, bag type and all its interactions, as well as the interaction of treatment and time, were not significant. Treatment: $P < 0.0001$. Time: $P = 0.04$. Data shown are least squared means of polymers in F57 and R510 bags in duplicate (LSM \pm SEM)53

Figure 2.2. Projected degradation of forage controls alfalfa hay and corn silage and polymer treatments PHA, PBSA, Blend, and Filament. Projected degradation was determined from calculated disappearance kinetics parameters A and k as determined from the potential degradability equation (Table 2.1). Kinetics parameters plotted for polymer treatments are mean values of those fermented in F57 and R510 bags in duplicate. Kinetics parameters plotted for forage controls are mean values of those fermented in only F57 bags in duplicate as bag type was significant.....54

Figure 2.3 Melting temperature of PHA, PBSA, Blend, and Filament treatments after fermentation in rumen fluid for 0, 24, 96, and 240h in F57 bags. Melting temperature ($^{\circ}\text{C}$) of treatments were determined from the first endothermic peak in the first heating scan. When comparing nurdles, Treatment: $P < 0.01$, Time: $P < 0.01$, Treatment x Time: $P < 0.01$. When comparing Blend and Filament treatments, Treatment: $P = 0.01$, Time: $P = 0.05$, Treatment x Time: $P = 0.23$. Data are shown as least squared means of treatments in duplicate (LSM \pm SEM).55

Figure 2.4 Onset degradation temperature of PHA, PBSA, Blend, and Filament treatments after fermentation in rumen fluid for 0, 24, 96, and 240h in F57 bags separated by: A) PHA component and B) PBSA component. Onset thermal degradation temperature ($^{\circ}\text{C}$) of treatments were determined to be the temperature at which 5% of the initial mass degraded. For the PHA components, time was the only significant parameter. Time: $P < 0.0001$. For the PBSA components: Treatment: $P < 0.0001$, Time: $P < 0.0001$, Treatment x Time: $P < 0.0001$. Data are shown as least squared means of treatments in duplicate (LSM \pm SEM).....56

Figure 3.1 In situ disappearance (%) of proprietary polyhydroxyalkanoate-based polymer (PHA), poly(butylene succinate-co-adipate) (PBSA), PBSA:PHA (90% wt PBSA, 10% wt PHA) (Blend), and low-density polyethylene (LDPE) films incubated in the rumens of three cannulated, non-lactating Holsteins for up to 150d. Treatment: $P < 0.001$. Time: $P < 0.001$. Treatment x Time: $P < 0.001$. Data are shown as least squared means of nine replicates distributed among three cows (LSM \pm SEM).....77

Figure 3.2 Degradation of PHA modeled by the predicted digestibility equation.....78

Figure 3.3 Visual assessment of PHA, PBSA, Blend, and LDPE films incubated in the rumens of three cannulated, non-lactating Holstein cows for up to 150d.....79

Figure 3.4 Residue length (mm) of PHA, PBSA, Blend, and LDPE films incubated in the rumens of three cannulated, non-lactating Holstein cows for up to 150d. Treatment: $P < 0.0001$. Time: $P < 0.0001$. Treatment x Time: $P < 0.0001$. Data are shown as least squared means of nine replicates distributed among three cows (LSM \pm SEM).....80

Figure 4.1. Average daily feed intake determined on a weekly basis, as fed, of Holstein bull calves given either empty gelatin capsules (Control), 13.6g of a Poly(butylene succinate-co-adipate) and Polyhydroxyalkanoate melt-blend (Blend), or 13.6g of Low-density Polyethylene for 30d, separated by average daily grain intake (**A**) and average daily hay intake (**B**). For grain intake, Treatment: $P = 0.28$; Day: $P < 0.01$; Treatment x Day: $P = 0.60$. For hay intake, Treatment: $P = 0.73$; Day: $P < 0.01$; Treatment x Day: $P = 0.82$. Data are shown as least squared means of treatments within time (LSM \pm SEM)114

Figure 4.2. Weekly body weights of Holstein bull calves given Control, Blend, or LDPE treatments for 30d. Treatment: $P = 0.69$. Day: $P < 0.01$. Treatment x Day: $P = 0.15$. Data are shown as least squared means of treatments within time (LSM \pm SEM).....115

Figure 4.3 Papillae length in the caudal ventral (CaV) and cranial dorsal (CrD) regions of the reticulorumens of Holstein bull calves after 30d of Control, Blend, or LDPE treatment administration. Treatment: $P = 0.19$. Region: $P < 0.0001$. Treatment x Region: $P < 0.001$.
^{a,b}Different letters indicate differences between treatments within epithelium region.
*Indicates a significant difference between regions within treatments. Data are shown as least squared means of treatments within region (LSM \pm SEM).....116

Figure 4.4 Characterization of polymer residues within rumen contents of Holstein bull calves given either Blend or LDPE treatment for 30d, separated by: **A**) estimated total polymer accumulation, and **B**) average polymer length. For both parameters, Treatment: $P < 0.0001$. ^{a,b}Different letters indicate differences between treatments. Data are shown as least squared means of treatments (LSM \pm SEM)117

INTRODUCTION

It is currently estimated that 20% of cattle contain foreign materials within the reticulorumen, which are primarily composed of plastic (Mushonga et al., 2015, Mekuanint et al., 2017). Single-use agricultural plastics for feed storage, such as bale netting or twine, may accidentally be incorporated into cattle feed. As largely indiscriminate feeders, cattle may then ingest these plastics. Polyethylene-based plastics are indigestible and, when consumed, tend to form large ball-like masses that press tightly against rumen epithelium during contractions (Priyanka and Dey, 2018). This phenomenon may lead to blockage of the gastrointestinal tract, reduced feed intake, weight, and production, as well as erosion and ulceration of epithelium, stunting of papillae, and reduced ruminal motility (Abd Al-Galil and Akraiem, 2016, Mekuanint et al., 2017, Otsyina et al., 2017, Harne et al., 2019, Mahadappa et al., 2020). A solution to reduce the incidence of plastic impaction in cattle is the development of biodegradable feed storage materials that will either be completely digested by the animal or degrade into smaller fragments that may pass through the gastrointestinal tract with no harm to the animal.

Materials of interest are biodegradable polymers, particularly polyhydroxyalkanoates (PHA) and biodegradable poly(butylene succinate-*co*-adipate) (PBSA). These polymers have mechanical properties similar to polyethylene with the added benefit of being degradable in the environment by select bacteria largely from the Proteobacteria phylum and abiotic factors (Lucas et al., 2008, Nakajima-Kambe et al., 2009, Liu et al., 2010, Wu, 2012, Shah et al., 2013, Vigneswari et al., 2015, Roohi et al., 2018, Salomez et al., 2019). Given that one of the most abundant phylum within the

rumen microbiome are Proteobacteria, along with an abundance of cellulolytic bacteria (Bryant, 1959, Van Soest, 1994, Oyeleke and Okusanmi, 2008), it is possible that the rumen environment may foster the degradation of PHA and PBSA.

The objectives of these studies were to evaluate the degradability of PHA and PBSA materials in the reticulorumen via in vitro, in situ, and in vivo methods. Our hypothesis was that these materials would degrade in the rumen and that a melt-blend of PHA and PBSA may degrade faster than its individual components.

CHAPTER 1

LITERATURE REVIEW

1. Agricultural Plastics and Ruminant Health

Due to population expansion, the use of plastics continues to exponentially increase across the globe. In 2017, 348 million tons of plastics were produced, of which agricultural plastics accounted for at least 2% (Jansen et al., 2019). This amounts to nearly 7 million tons of agricultural plastics, which are primarily single-use and rarely recycled. Most agricultural plastics are either landfilled or burned, which may pollute the air with toxic chemicals and release microplastics into soil. These agricultural plastics are mostly comprised of animal feed storage materials such as bale twine, bale netting, bale wrap, and silage bags (Janke and Trechter, 2015). These materials build up quickly on the farm and can get caught in equipment, pollute waterways, or produce microplastics in soil.

Aside from disposal methods and potential environmental impacts, plastics are proving to be detrimental to animal health via their ingestion. The most common manner is through the incorporation of plastic feed storage materials in cattle rations. This could be due to directly providing fodder with the attached materials or grinding bales with intact netting. If ingested, these materials are not digestible (Klein and Dahlen, 2014). Some reports found approximately 20% of domestic cattle, sheep, and goats at slaughter have foreign bodies within the rumen, of which, 50 to 60% are plastic materials. In these reports, amounts of foreign bodies found within animals were proportional to age and inversely proportional to body condition score (Mushonga et al., 2015, Mekuanint et al., 2017). Reports like these have not widely been conducted in the United States, but

several case studies indicate ingestion of large amounts of plastics in domestic ruminants is becoming more prevalent.

Once plastic materials are ingested by cattle, they either remain in the rumen or, if small enough, are passed through the digestive tract. A feeding study offered beef cattle forage that was chopped with intact net wrap and found that after 7 months of daily feeding that animals retained 47% of the offered plastic in the rumen in large ball-like masses (Pizol et al., 2017). It is postulated that by a cow's third lactation, it could have upwards of 7kg of plastic trapped in the rumen. These data provide evidence of plastic accumulation in the digestive systems of cattle under normal operating conditions. This plastic build up could potentially lead to productivity and health issues in these animals.

Further reports on animals with masses of plastics within the rumen found associated gross and histopathological effects (Mekuanint et al., 2017, Otsyina et al., 2017). Dorper sheep containing plastics up to 1.5% of their body weight within the rumen demonstrated severe atrophy of body muscle and fat, erosion and ulcerations of ruminal papillae, mucosal congestion, thinning of the rumen wall, irregular disruption of epithelium, and increased mononuclear cell proliferation after 6 weeks of plastic presence. Though, these effects became apparent with plastic accumulation of just 0.5% of their body weight (Otsyina et al., 2017). Disruption of rumen epithelium and animal health was likely due to the pressure of the plastic against the rumen walls during rumination, impaction of the digestive tract, and blockage of the fermentative process. Plastic accumulation within the rumen could be deleterious to feed efficiency, weight gain, and milk production. No known studies have yet elucidated the relationships between plastic accumulation and long-term performance.

In addition to physiological effects within the rumen, plastic presence may compromise rumen health and efficiency via leakage of heavy metals. During commercial processing of plastic materials, plasticizers are incorporated which often contain phthalates and heavy metals that can be toxic to humans and animals (Oehlmann et al., 2009, Halden, 2010, Holmes et al., 2012). Heavy metals such as mercury, lead, cadmium, chromium, and copper were found to be significantly increased in body fluids and tissues by at least 85% in buffalo with plastic impaction (Mahadappa et al., 2020). The authors believe the saturation of plastic materials in rumen fluid causes heavy metals to leach from the materials themselves and accumulate. Increased heavy metal concentrations within rumen fluid were highly correlated with decreased rumen protozoal density and motility, and increased rumen pH, methylene blue reduction time, and sedimentation activity time in these animals. This indicates that heavy metals released by plastics in the rumen are deleterious to the health of the ruminal microbiome and negatively impact their fermentative ability of feeds. This could have serious implications for animal health and feed efficiency.

It is apparent single-use plastics such as bale twine and netting can have serious repercussions regarding animal health when consumed. Unfortunately, unlike with hardware disease in cattle, there is no easy treatment for plastic impaction in ruminants. The only solution at this point is a costly surgery to manually remove the plastic. Even after plastics are removed, it can take the animal several months to fully recover to their potential production (A and A. S. A, 2016). One solution to prevent the ingestion of non-digestible plastics in ruminants is to develop a biodegradable plastic alternative. Some biodegradable materials for net wrap are available for purchase, however, these are not

degradable within the digestive tract as they primarily rely on UV radiation in the environment to break down (Klein and Dahlen, 2014). Polyhydroxyalkanoates (PHA) are biodegradable materials that show promise as a plastic alternative as they are derived from bacteria and exhibit properties very similar to common plastics (Kadouri et al., 2005).

2. Polyhydroxyalkanoates

Polyhydroxyalkanoates are organic polymers with open ring chemical structures produced via fermentative processes within various microorganisms during unbalanced growth, in which select nutrient sources are deprived in the presence of an abundant carbon source (Shen et al., 2009, Mudenur et al., 2019). They accumulate within the cytoplasm of microorganisms as “discrete granules” of energy that can account for up to 90% of cellular dry weight (Jendrossek and Handrick, 2002). Polyhydroxyalkanoates are known to be produced by at least 75 genera of bacteria and fungi in a vast array of environments (Mudenur et al., 2019). The different classes of PHAs produced depend on the microorganism and carbon source they were sequestered from, and they structurally vary by their functional group ([Table 1.1](#)). Carbon chain lengths of the functional group classify PHAs into two categories, short-chain length (up to five carbons) and medium-chain length (six to sixteen carbons), which differ in their mechanical properties and their degradability by various microorganisms (Shen et al., 2009, Roohi et al., 2018). The simplest and most abundantly studied PHA is poly(3-hydroxybutyrate) (PHB), a short-chain length PHA with a methyl for its functional group (Roohi et al., 2018).

2.1 Commercial Production

Commercial synthesis of these PHAs varies dependent upon the selected microorganism and carbon source, but the overall process is the same: fermentation, isolation, and purification (Shen et al., 2009). There are three types of fermentation methods that each have their advantages and disadvantages depending on the chosen microorganism: batch, fed-batch, and continuous-batch. The batch method is a closed system in which the substrate and inoculum are added to the fermenter in a batch. In the fed-batch method, the substrate is added gradually as it is consumed. The continuous-batch method is an open system in which fresh media is added at regular intervals (Mudenu et al., 2019). At this point, PHA is accumulated intracellularly within microorganisms. Intracellular PHA is insoluble and in an amorphous elastomeric state coated in a phospholipid membrane (Barnard and Sanders, 1988, Fuller et al., 1992, Horowitz and Sanders, 1994). To isolate and purify the intracellular PHA an “enzyme cocktail” and a solvent extraction method are used. The enzyme method removes proteins, nucleic acids, and cell walls via proteases, nucleases, and lysozymes to produce neat granules of PHA. The granules are dissolved in warm chloroform and then precipitated out using methanol for purification (Shen et al., 2009). The resulting product is extracellular PHA, which is structurally different from amorphous, intracellular PHA. Extracellular PHA is partially crystalline, which gives it its plastic-like quality (Jendrossek and Handrick, 2002).

Polyhydroxyalkanoates can be used as plastic substitutes by themselves, however, they tend to be expensive and mechanically brittle (Shen et al., 2009). Copolymers or blends can be developed by blending monomer units with other PHAs or biodegradable

polymers such as poly(butylene succinate-*co*-adipate) (PBSA). Blending PHAs with PBSA improves mechanical properties that may make PHAs more suitable for market while maintaining their biodegradability (Twarowska-Schmidt and Tomaszewski, 2008, Jompang et al., 2013). However, the extent of improvement largely depends on the type, length, and distribution of comonomer units (Shen et al., 2009) (Twarowska-Schmidt and Tomaszewski, 2008, Jompang et al., 2013).

Currently, the largest hindrance to mass-producing PHAs to replace common plastics is the cost of production and inefficiency of PHA synthesis by microbes. Several studies are in place to determine cheaper carbon sources, such as waste products from various industries, and to identify bacterial strains with the more efficient production of PHAs. As efficient bacterial strains and cheaper carbon sources are identified, it is estimated that PHA production will become more suitable and will increase to satisfy the strong demand for environmentally friendly products (Chen et al., 2020).

2.2 Microbial Degradation

Intracellular PHAs are degraded by the accumulating microorganism via intracellular PHA depolymerases, whereas extracellular PHAs are degraded by extracellular PHA depolymerases in the environment. Different depolymerases are required because intracellular and extracellular PHAs are structurally different; intracellular PHA has phospholipids and proteins on the surface layer whereas extracellular PHA is partially crystalline (Jendrossek and Handrick, 2002).

Depolymerases bind to the substrate and hydrolyze the material into water-soluble monomers and/or oligomers specific to individual PHAs and blends thereof, which are then further mineralized by extracellular enzymes to water and carbon dioxide and/or

methane if not taken up by other microbes (Roohi et al., 2018). For example, PHB is degraded into 3-hydroxybutyrate, poly(3-hydroxyvalerate) (PHV) is degraded into 3-hydroxyvalerate, and poly(3-hydroxybutyrate-co-3-hydroxyvalerate) (PHBV) is degraded into both monomers (Liu et al., 2019).

Microorganisms that produce extracellular PHA depolymerases can be isolated from various ecosystems like soil, compost, aerobic and anaerobic sewage sludge, fresh and marine water, the gastrointestinal tracts of marine species, and even the air (Liu et al., 2010, Roohi et al., 2018). Genres most associated with producing extracellular depolymerases include *Cupriavidus*, *Alcaligenes*, *Comamonas*, and *Pseudomonas* (Vigneswari et al., 2015). The biodegradability of PBSA has been less widely evaluated, yet studies have demonstrated enzymatic degradation by *Roseateles depolymerans* (Shah, 2013), *Azospirillum* sp. (Wu, 2012), and *Leptothrix* sp. (Nakajima-Kambe et al., 2009), among others. However, these enzymes are not always active, even in the presence of these materials. When there are suitable carbon sources available, like glucose or organic acids, some microorganisms will exhaust those sources before expressing depolymerases. Additionally, expression of depolymerases can be hindered by pH and the presence of fatty acids (Jendrossek and Handrick, 2002). Depolymerase activity of microorganisms is also selective to short-chain length versus medium-chain length polymers, though some microorganisms will produce both classes of depolymerases. There is limited knowledge on the breadth of degradation of these biodegradable polymers by microorganisms, and further studies are warranted due to extensive variability within the literature (Jendrossek and Handrick, 2002).

2.3 Abiotic Degradation

In combination with microorganisms, abiotic factors play a major role in the biodegradation of PHAs and PBSA. However, these factors are more synergistic to the biodegradation process rather than the main drivers. Thus, they may simply initiate or streamline the biodegradation process by microorganisms by fragmenting polymers to increase the surface area available for microbial attachment (Albertsson et al., 1994, Hakkarainen, 2001, Salomez et al., 2019). Abiotic factors include mechanical stress, light, temperature, chemicals, and spontaneous hydrolysis (Lucas et al., 2008). In addition to biotic and abiotic factors within an environment, the physical presence of the polymer, such as crystallinity, molecular weight, and thickness, determine the biodegradability of polymers (Mergaert et al., 1992, Jendrossek and Handrick, 2002, Lucas et al., 2008, Su Yean et al., 2017).

2.4 Applications

Polyhydroxyalkanoates and PBSA-based materials are currently in use to replace some single-use plastics to reduce plastic overloading and the production of microplastics in the environment. They are used in the production of food packaging and agricultural plastics such as mulch films, agricultural nets, and agricultural grow bags (Amelia et al., 2019, Rafiqah et al., 2021). In the biomedical field, PHB is used to develop implanted devices such as screws, plates, films, and biodegradable sutures for procedures such as articular cartilage repair, meniscus repairs, and cardiovascular patch grafting (Roohi et al., 2018, Mudenur et al., 2019). Similarly, PBSA is used in tissue engineering and bone grafting (Rafiqah et al., 2021). Polyhydroxyalkanoates are used in pharmacology as a sustainable delivery method of anti-inflammatory drugs via microcapsules due to their

slow rate of degradation in tissues (Kalia et al., 2019). Further, PHAs have been investigated as biocontrol agents given their short-chain fatty acid oligomers and monomers upon degradation that prevent the growth of opportunistic pathogens (Gowda and Shivakumar, 2019). As such, PHAs and PBSA are considered body-safe in humans. However, no known studies evaluated ingestion of PHA in humans.

Though limitedly, PHAs have been investigated for animal use, however, PBSA has yet to be investigated. Separate studies determined PHB's ability to act as an anthelmintic in European sea bass, Chinese mitten crabs, and brine shrimp. It was found that, when supplemented with PHB either before or during parasitic challenges, these species had better survival rates proportional to fed PHB concentrations (Defoirdt et al., 2007, De Schryver et al., 2010, Sui et al., 2012). Animals like European sea bass and Chinese mitten crabs additionally had improved feed efficiency, improved growth rates, a reduction in gut pH, and a greater change in gut microbial populations (De Schryver et al., 2010, Sui et al., 2012). As such, it was determined that these animals were able to degrade PHB, and short-chain fatty acids like 3-hydroxybutyrate were released. Degradation of PHB was likely via PHB-degrading bacterium that produced depolymerases, as isolated from the gastrointestinal tracts of sea bass, sturgeon, and river prawns (Liu et al., 2010). Though these studies are promising indicators that these biodegradable materials may degrade in digestive tracts, they did not specifically evaluate polymer degradation and only focused on monogastrics. There is limited information on the interaction of biodegradable polymers with the ruminant gastrointestinal tract to postulate degradation kinetics for this group of animals.

To begin to postulate the true ability of ruminants to degrade PHA and PBSA and potential impacts on animal health, their unique digestive tract and fermentative ability need to be reviewed.

3. Ruminant fermentation

Ruminants are herbivorous animals that include cattle, sheep, goats, deer, antelope, and many other grazing mammals. These animals have a unique digestive tract, and digestion process, to accommodate their forage-based diets. The digestive tract in full includes the mouth, esophagus, stomach, small intestine, large intestine, colon, and anus. Unlike monogastrics, ruminants have a four-compartment stomach where each compartment serves a unique function: rumen, reticulum, omasum, and abomasum. The rumen serves as a fermentation vat where most nutrient absorption occurs. The reticulum controls the passage of feed particles by size to the omasum and traps foreign bodies. The omasum is the largest site of water absorption with limited nutrient absorption. The abomasum is the glandular stomach that chemically digests remaining materials before transference to the small and large intestines (Van Soest, 1994). The focus of this review will be on the rumen.

3.1 Rumination

When ruminants ingest feed, it is masticated, mixed with saliva, and swallowed as a bolus that moves down the esophagus and deposits into the rumen and reticulum. However, upon first ingestion feed is not fully masticated. Instead, ruminants undergo the process of rumination. Rumination is the postprandial regurgitation of ingesta which is re-masticated, mixed with saliva, and swallowed as a bolus (Van Soest, 1994). This process is stimulated by epithelial sensors and is closely aligned with ruminal

contractions that help eject longer fibers through the esophagus. Rumination allows ingesta to further be broken down into smaller particles, enriches fibrous content, and reintroduces saliva to buffer acids introduced to the rumen during fermentation (Van Soest, 1994). The process is cyclical and will continue for a select feed until it is reduced enough to pass through the reticulum and proceed through the digestive tract; though, the exact size of material needed to pass through to the omasum has not been determined and may be variable among ruminants. Overall, more efficient fermentation and energy utilization can occur from this process.

3.2 Fermentation

Ruminants rely on their symbiotic relationship with microbes since they do not produce the necessary enzymes to break down the fibrous materials they ingest. Ruminal fermentation is the process by which anaerobic and facultative microorganisms within the rumen break down and ferment low quality fibrous ingesta into utilizable energy sources for the animal (Castillo-González et al., 2013). The products of microbial fermentation in the rumen account for more than 70% of the ruminant's total energy supply. The microbial population consists of a wide diversity of microbes with approximately 10^{11} cells/mL bacteria, 10^6 cells/mL protozoa, and 10^4 cells/mL fungi, and these relative populations change within animals to accommodate for changes in environment and diet (Van Soest, 1994, Castillo-González et al., 2013, Owens and Basalan, 2016). Further, enzymatic expression of these microbes differs within animals fed different diets (Ali and Tirta, 2001).

Ruminal microorganisms are often grouped by the substrates they hydrolyze and ferment. These microorganism groups include cellulolytic, hemicellulolytic, amylolytic,

lipolytic, lactate-degrading, and pectin-degrading bacteria and protozoa, and methanogens (Van Soest, 1994, Castillo-González et al., 2013, Owens and Basalan, 2016). Microbes within the rumen form a unique, interwoven system to hydrolyze and ferment ingesta into usable products for the animal.

Ingesta exists in a polymeric form and must first be hydrolyzed into monomeric substrates to be used by primary fermenters. Carbohydrates like starches, hemicellulose, cellulose, and sugars are hydrolyzed into glucose, lipids are hydrolyzed into glycerol and fatty acids, and proteins are hydrolyzed into amino acids (Owens and Basalan, 2016). Primary fermenters like cellulolytic or proteolytic bacteria can ferment these monomers, and the byproducts of their fermentation are the substrates for secondary fermenters like methanogens and acetogens. The metabolic pathways of both primary and secondary fermenters are interwoven so that end products or intermediates of one microbe's fermentation may be the substrate of another's fermentation. This achieves the end products of fermentation necessary for ruminant nutrition (Castillo-González et al., 2013).

The end products of fermentation are mainly short-chain volatile fatty acids (VFA), carbon dioxide, and methane. The primary VFAs produced are acetic, propionic, and butyric acid. Other organic acids may be produced as intermediates like lactic and succinic acid but these are further fermented into the principle organic acids listed. The proportion of these acids within the rumen is dependent on the diet of the animal and methanogen populations. Trace amounts of hydrogen gas and nitrogen gas are also produced (Van Soest, 1994, Castillo-González et al., 2013, Owens and Basalan, 2016).

3.3 Fate of Fermentative End Products

Gases, particularly methane, are not further utilized by the animal. They are an energy loss as they are either eructated or passed through the rumen epithelium into the blood stream to later be exhaled (Van Houtert, 1993, Van Soest, 1994).

Volatile fatty acids are the main fermentative byproduct that animals use for energy. These acids are removed from the rumen via a gradient across the rumen epithelium. Higher concentrations of VFAs and decreased pH within the rumen compared to the portal blood favor their absorption (Van Soest, 1994). Not all organic acids reach the blood unaltered, and metabolism may begin within the epithelium. Butyrate is largely converted to the ketone body 3-hydroxybutyrate within the rumen epithelium. Propionate can be converted to lactate and other metabolites or oxidized to carbon dioxide. However, this conversion is minor, and the majority of propionate metabolism occurs later within the liver. Acetate is largely left unaltered (van Houtert, 1993). In the liver, remaining butyrate is converted to 3-hydroxybutyrate and acetoacetate to be used for energy, gluconeogenesis, or lipogenesis. Propionate is almost completely metabolized and used as a substrate for energy and gluconeogenesis. Though acetate again is largely left unmetabolized, acetate in the peripheral blood is taken up by various tissues throughout the body and metabolized for energy or lipogenesis (Van Soest, 1994).

3.4 Maintenance of Rumen Environment

The rumen environment must be maintained to ensure proper conditions for microbial growth and metabolism. If the fermentative environment is disrupted, deleterious effects could occur regarding animal health and production. Several factors need to be maintained within the rumen, but one of the most important is pH. Ruminal

microbes, particularly cellulolytic, operate at an optimal pH of about 6.7, and often a pH below 6.2 will inhibit digestion rates (Van Soest, 1994, Castillo-González et al., 2013). Most metabolic diseases occur concurrently with decreased pH due to dietary changes. Acidosis is the most prevalent one and is often associated with a stark increase as grain proportions increase within the diet. This quickly increases the production of acetate in the rumen and decreases the pH, which in turn reduces the microbial population and can impact ruminal epithelium. Pending the extent of ruminal disruption, this could have grave consequences on animal production. However, the ruminal population will accommodate the dietary change after a time of about 8 weeks (Van Soest, 1994). Given the extended period required for adaptation, it is advantageous to have a consistent diet that may slowly change to accommodate changing energy requirements.

Ruminants have inherent pathways to combat increases in pH due to fermentation. Ruminal pH is largely regulated by the introduction of saliva from ingestion and rumination, the removal of VFAs, and the production of methane (Van Soest, 1994). As such, animals must be fed a consistent diet with long fibers throughout the day. This will promote apt rumination and consistently introduce saliva to the rumen to act as a buffer. Microbes will then be able to metabolize plentiful nutrients to maintain their health. This will produce more VFAs and increase their concentration within the rumen compared to the portal blood and promote their absorption across the epithelium. The maintenance of methanogens is also important within the rumen. Methanogens reduce carbon dioxide by the addition of free hydrogen ions, and effectively increase ruminal pH (Castillo-González et al., 2013). Lastly, plenty of water needs to be offered throughout the day to keep ingesta moving throughout the tract. This allows for efficient rumination and

eructation of gases and promotes greater feed intake of the animal. Many factors play into maintaining the rumen environment, and it is important to understand them and their balance to promote the most effective fermentation.

4. Potential ruminant degradation of biodegradable polymers

There is much that we do not know considering the ability of ruminants to effectively digest PHAs and blends with PBSA due to the paucity of the literature. To date, only one study directly evaluated the ingestion of PHAs by ruminants. Forni et al. (1999) studied the degradation and metabolic energy utilization of polycaprolactone (PCL) and PHBV in sheep. Results indicated that incorporation of these polymers in the diet resulted in reduced digestibility of organic matter and increased energy losses in fecal matter. However, these results were likely produced due to the method by which biopolymers were incorporated into the diet. As fine powders, the polymers were likely not retained in the rumen long enough for present enzymes to bind and hydrolyze the material into utilizable fatty acids. Therefore, the material escaped the digestive tract largely intact and the available energy was lost in the feces.

To determine the ability of ruminants to digest PHAs and PBSA, and the worth of investigating this material as a potential replacement for bale twine or netting materials, many factors must be considered. The presence of depolymerase-producing microbes within the rumen, potential ecological changes, the overall impact of fermentative products on the animal, and histological impacts from the physical material being ingested should be contemplated.

4.1 Rumen Ecology

It is presently uncertain if rumen fluid typically contains a bacterium that produces depolymerases; however, it is highly likely. The ability of bacteria to produce depolymerases for these materials is not necessarily rare, but rather the scope has not been fully explored. Extracellular depolymerases are produced by a wide assortment of microbes: gram-positive, gram-negative, aerobic, facultative, and anaerobic bacteria alike (Nakajima-Kambe et al., 2009, Wu, 2012, Shah et al., 2013b). Given that the rumen microbial population has a high propensity to degrade cellulose's crystalline structure and Proteobacteria is one of the most abundant phyla within the rumen, species within Proteobacteria are likely capable of degrading PHA and PBSA materials.

One study evaluated the presence of PHA-degrading bacteria in rumen fluid. Budwill et al. (1996) evaluated in vitro anaerobic degradation of PHB within rumen fluid and found no apparent degradation indicated by changes in methane production compared to a control. However, clearing zones formed on PHB overlay plates with serial dilutions of rumen fluid that indicated hydrolysis of PHB. A *Staphylococcus* sp. that produced a very active depolymerase was later isolated from those colonies (Budwill et al., 1996). However, a generalization for all ruminants' ability to host PHA-degrading bacteria cannot be determined at this time due to this study's utilization of rumen fluid collected from a single dairy cow.

Though ruminants likely contain ruminal microbes from the Proteobacteria phyla with the ability to produce depolymerases that degrade PHA and PBSA, it is also possible that these enzymes are not consistently, or even initially, expressed. It has been observed that some bacteria only express depolymerases when there are no other carbon sources

available to them (Jendrossek and Handrick, 2002). It may take a particular proportion of material within the rumen compared to regular ingesta for the expression of depolymerases to occur in vivo. This area has yet to be explored and an estimated threshold cannot be determined at this time.

It is also plausible that PHA and PBSA presence in the rumen will change the microbial ecology. The microbial population naturally changes as the diet of the animal changes, usually taking about 8 weeks to regulate (Van Soest, 1994). If ingested and not first degraded, the “plastic” will bundle together at the bottom of the rumen and slowly accumulate over time, taking a larger proportion of ruminal contents. This accumulation may be necessary to initially activate the enzymatic expression of these depolymerases. Pending the rate of degradation, the polymer material may always be present and support the growth of bacteria that degrade the material. This will make it so that animals previously exposed to the plastic replacement will be better suited to degrade it. Though, if the rate of degradation is not sufficient, serious accumulation could occur and begin to impact animal health and production.

4.2 Rate of Degradation

Should the rumen environment degrade PHA and PBSA, it is difficult to estimate “true” digestive rates and the time needed to achieve maximum degradation due to the lack of literature and high variability within it. Previous literature has not extensively evaluated the rates of degradation of PHAs or PBSA in the digestive tracts of animals. Natural degradation experiments have mostly been conducted with PHB and its copolymers in soil, compost, sewage sludge, or river water. Various degradation rates have been achieved across and within these environments due to differences in

environment pH, temperature, and microbial communities and the size, shape, and composition of materials tested.

One study buried 1cm² film samples of PHB in soil (pH 7.3, 30°C) and achieved 60% degradation after six weeks (Altaee et al., 2016). Conversely, Corrêa et al. (2008) observed only 2.5% degradation of PHB films after five months under soil (pH 8.2, 25°C). Bucci et al. (2007) conducted a study on food packaging prototypes made of PHB. Samples had surface area of 900mm², thickness of 1.24mm, and weighed 1.34g. Within 90 days, all samples in organic residues for composting, sewage, or anaerobic septic tank were completely degraded.

Given the elevated temperatures (39 to 40°C), neutral to acidic pH (5.5 to 7.0), higher microbial population, and motility within the rumen, it is hypothesized that degradation rates achieved in the rumen may be increased compared to these trials. However, the digestive rate is highly dependent on the form and density of the material and blending of copolymers, where thinner materials with greater surface area and lower crystallinity are more favorable (Su Yean et al., 2017). It is currently estimated that degradation of these materials within the rumen will take anywhere from three to six months pending form and crystallinity.

4.3 Effect of Byproducts

No toxic effects have been demonstrated from the ingestion of PHAs by marine monogastrics (Defoirdt et al., 2007, De Schryver et al., 2010, Sui et al., 2012). Further, no toxic effects have been observed within ruminants, though PHBV was largely not degraded (Forni et al., 1999).

We hypothesized that ruminants already have the pathways to metabolize the byproducts of PHA and PBSA degradation. Polyhydroxyalkanoate polymers are hydrolyzed into oligomeric and monomeric short-chain fatty acids, carbon dioxide, and water, which are the common products of microbial fermentation within the rumen. Specifically, if PHB were to be ingested by the ruminant, it is presumed that microbes would hydrolyze the material into 3-hydroxybutyrate, carbon dioxide, and water. Butyrate is one of the primary VFAs produced by the fermentation of fibrous ingesta and is taken up into rumen epithelium according to a concentration gradient and metabolized into 3-hydroxybutyrate. The 3-hydroxybutyrate from PHB is likely to be taken up with butyrate into the rumen epithelium to the blood. It may then be metabolized in the liver for energy, gluconeogenesis, or lipogenesis. Carbon dioxide would either be eructated, diffused into the blood, or reduced to methane by methanogens to increase pH. As such, it may be beneficial for the animal to ingest small portions of PHA materials for the added supply of energy.

Alternatively, PBSA degrades into its monomeric constituents, 1-4, butanediol, succinate, and adipate (Shah et al., 2013a). Butanediol is rapidly metabolized to gamma-hydroxybutyrate, which in turn is metabolized into succinate (Irwin, 1996). Succinate is naturally present within animals and utilized in several pathways such as the elimination of reactive oxygen species, endocrine, and paracrine modulation, and inflammation, among others in rat and mice models (Tretter et al., 2016). In rats, adipate was found to have a LD₅₀ of more than 5,000mg/kg and be metabolized to urea, glutamic acid, lactic acid, beta-ketoadipic acid, and citric acid, which may indicate its metabolism via beta-oxidation (Kennedy, 2002, Auffret et al., 2017). Therefore, ruminants should have natural

mechanisms to metabolize and dispose of end products of PBSA biodegradation within the rumen.

Possible issues regarding degradation byproducts of PHA and PBSA would be dependent on the rate of degradation. If materials are degraded too quickly, pH within the rumen could decrease below the optimum level due to the rise of short-chain fatty acids within the rumen, and this could potentially lead to a reduction of pH-sensitive bacteria, such as those that degrade cellulose. This may make ruminants more susceptible to acidosis (Li et al., 2017). Additionally, in the case of PHB, elevated levels of 3-hydroxybutyrate within the plasma of dairy cows at or above 1.2mmol/L have been associated with ketosis and negative effects on animal health and production (Duffield et al., 2009). However, it is presently assumed that degradation will occur slowly over months. Thus, pH changes from hydrolysis products are likely to be combatted by the rumen's natural buffers and methanogens. Additionally, it is assumed that 3-hydroxybutyrate levels will not increase so much as to indicate ketoacidosis.

4.5 Potential Histopathological Effects

Regardless of the proposed material's ability to degrade within the rumen, the effect of the plastic physically present within the rumen should be considered. If degradation of PHA and PBSA is slow or delayed, the presence of a biodegradable polymer-based netting will likely have the same effects as typical plastic net wraps if ingested (Otsyina et al., 2017, Pizol et al., 2017). The degree of effect, however, will be influenced by the ability of the rumen environment to foster degradation of the material and the rate at which it is degraded. If ingested, the netting is likely to matt together in a ball-like mass at the bottom of the rumen that will press tightly against the rumen

epithelium with contractions (Otsyina et al., 2017). This could disrupt the papillae's ability to absorb VFAs and other nutrients. Additionally, too much plastic buildup could eventually lead to decreased feed intake and muscle and fat atrophy in the most extreme cases. However, the degradation of these products is highly likely to reduce or eliminate these effects.

It is presently assumed that ruminants in United States production systems exposed to this material will only ingest small portions at a time (Pizol et al., 2017). Matting is not thought to occur immediately, and the small filaments may be degraded as they are introduced. As the material is degraded, filament length will decrease, and potential matting size will be reduced as smaller fragments move through the digestive tract. This will lessen the effect of plastic pressure against epithelium and the likelihood of feed intake reduction or tissue atrophy occurring. If degradation of the material occurs, it is hypothesized that the histological effect on the animal will be significantly lower compared to an animal ingesting the typical plastic bale twine or netting.

5. Conclusion

The growing use of single-use plastics in agriculture and their continuous accumulation has negatively impacted ruminants via ingestion and entanglement in the rumen. As such, this phenomenon is rapidly becoming the new hardware disease of the century. One solution is to replace common plastics with PHAs, biodegradable materials synthesized by bacteria in the presence of an abundant carbon source during nutrient depletion. Blending these materials with biodegradable PBSA may improve their mechanical qualities and make them more suitable for market. These materials can be degraded by a broad range of microbes, though the entire scope is still under

investigation. The ability of ruminants to degrade these materials if ingested has hardly been investigated, and further trials are warranted to determine the suitability of the products to replace agricultural plastics. Given that ruminants ferment their food via cellulolytic and other enzyme-producing microbes, it is predicted that ruminants are capable of digesting PHAs and PBSA via microbial production of extracellular depolymerases. These materials are hydrolyzed into their monomeric constituents, carbon dioxide or methane, and water. It is then assumed that these hydrolysis products will be metabolized by the ruminant via preexisting metabolic pathways and provide additional sources of energy for the animal. Presently, there is a limited concern for potential animal health or production impacts due to the ingestion of PHAs and PBSA compared to current plastic ingestion. However, this is highly dependent on the rate of degradation of the material within the rumen. Studies are warranted to determine the ability of the rumen environment to degrade these materials, and the rate of their degradation to understand the potential health and production implications of animals ingesting these products.

6. Tables

Table 1.1. Short-chain and medium-chain length polyhydroxyalkanoates, as adapted by Tan et al. (2014).

Classification	Polyhydroxyalkanoate	Carbons	R group
Short-chain length	Poly(3-hydroxybutyrate)	4	Methyl
	Poly(3-hydroxyvalerate)	5	Ethyl
Medium-chain length	Poly(3-hydroxyhexanoate)	6	Propyl
	Poly(3-hydroxyheptanoate)	7	Butyl
	Poly(3-hydroxyoctanoate)	8	Pentyl
	Poly(3-hydroxynonanoate)	9	Hexyl
	Poly(3-hydroxydecanoate)	10	Heptyl
	Poly(3-hydroxyundecanoate)	11	Octyl
	Poly(3-hydroxydodecanoate)	12	Nonyl
	Poly(3-hydroxytridecanoate)	13	Decyl
	Poly(3-hydroxytetradecanoate)	14	Undecyl
	Poly(3-hydroxypentadecanoate)	15	Dodecyl
	Poly(3-hydroxyhexadecanoate)	16	Tridecyl

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CHAPTER 2

Digestibility kinetics of polyhydroxyalkanoate and poly(butylene succinate-*co*-adipate) after in vitro fermentation in rumen fluid

1. Abstract

Using polyhydroxyalkanoate (PHA) and poly(butylene succinate-*co*-adipate) (PBSA) materials for feed storage products such as bale netting could reduce the incidences and effects of plastic impaction in ruminants. The objective of this study was to determine in vitro, ruminal degradability and kinetics of biodegradable polymers and blends. A proprietary PHA-based polymer, PBSA, PBSA:PHA melt blends, and forage controls were incubated in rumen fluid for up to 240h. Mass loss was measured after each incubation time, and digestion kinetic parameters were estimated. Thermogravimetric and differential scanning calorimetry analyses were conducted on incubated samples. Data were analyzed with PROC MIXED in SAS and adjusted by Tukey's method to determine least squared differences between polymer treatments, incubation time, and the interaction. Generally, across treatments, mass loss was significant by 96h with a minimum increase of 0.25% compared to 0h but did not change thereafter. Degradation kinetics demonstrated that polymer treatments were still in the exponential degradation phase at 240h with a maximum disappearance rate of 0.0031%/h. Melting temperature increased and onset thermal degradation temperature decreased with incubation time, indicating structural changes to the polymers. Based on these preliminary findings, the first stage of degradation occurs within 24h and PHA degrades slowly. However, further ruminal degradation studies of biodegradable polymers are warranted to elucidate maximum degradation and its characteristics.

2. Introduction

Animal feed storage materials such as bale twine, bale netting, bale wrap, and silage bags make up the majority of single-use agricultural plastics produced (Janke and Trechter, 2015). These materials not only pose a problem for the environment due to their poor disposal methods, but also are detrimental to cattle health. Cattle ingest these plastic materials when they are accidentally incorporated into the diet, and it is estimated that 20% of cattle contain foreign bodies with their rumen due to this (Mushonga et al., 2015, Mekuanint et al., 2017). These materials are indigestible and tend to form large ball-like masses that press tightly against rumen epithelium during contractions (Priyanka and Dey, 2018). This phenomenon not only impacts the rumen and leads to decreases in feed intake and productivity but may have long-term implications at low levels of accumulation. Ruminal epithelium becomes eroded with ulcerations and papillae shorten (Mekuanint et al., 2017, Otsyina et al., 2017, Harne et al., 2019), toxic heavy metals from plastics are leached into rumen fluid, blood, and tissues (Mahadappa et al., 2020), and rumen motility is reduced (Abd Al-Galil and Akraiem, 2016) in ruminants with plastic impaction that likely hinders rumen functionality and feed efficiency. It is imperative that biodegradable alternatives to polyethylene-based single-use agricultural plastics such as bale netting be sought for environmental sustainability and animal health. Materials of interest are bio-based polyhydroxyalkanoates (PHA) and biodegradable poly(butylene succinate-*co*-adipate) (PBSA).

Polyhydroxyalkanoates are aliphatic polyesters naturally produced via fermentative processes within various microorganisms during unbalanced growth when select nutrient sources are deprived in the presence of an abundant carbon source (Shen et

al., 2009, Mudenur et al., 2019). Polyhydroxyalkanoates have a vast array of applications due to their biodegradability in various environments, chemical diversity, insolubility in water, biocompatibility, and lack of toxins (Kadouri et al., 2005, Mudenur et al., 2019). Despite these benefits, PHAs are quite expensive and tend to be mechanically brittle. Polymer blends can be developed by blending PHA with other biodegradable polymers. Polymer blends with PHAs can have improved mechanical properties that may make them more suitable for market and maintain their biodegradability (Shen et al., 2009). Poly(butylene succinate-*co*-adipate) is a biodegradable polymer shown to improve elasticity and functionality of biodegradable polymers while reducing costs when blended (Twarowska-Schmidt and Tomaszewski, 2008, Jompang et al., 2013).

Both materials are readily in use for body-safe materials but have yet been sought for bale netting applications. Poly(3-hydroxybutyrate) (PHB), a particular PHA, has been of considerable interest in the biomedical field. Implanted devices such as screws, plates, films, and biodegradable sutures have been developed from these materials for procedures such as articular cartilage repair, meniscus repairs, and cardiovascular patch grafting. Disposable needles, syringes, sutures, surgical gloves, gowns, and more have been created from biodegradable polymers (Roohi et al., 2018, Mudenur et al., 2019). Likewise, PBSA is utilized in tissue engineering and bone grafting (Rafiqah et al., 2021).

Biodegradable polymers such as PHA-based polymers and PBSA can be degraded abiotically or biotically. Abiotic factors that contribute to breakdown include mechanical stress, temperature, and chemicals. These cause minimal degradation by themselves but are synergistic to more extensive biotic degradation and may initiate the biodegradation process (Lucas et al., 2008, Salomez et al., 2019). Polymers may degrade biotically via

formation of biofilms comprised of a wide assortment of microbes including gram positive, gram negative, aerobic, facultative, and anaerobic bacteria. These microbes produce extra-cellular depolymerases that enzymatically degrade polymers by first cleaving polymer chains, which are then assimilated and converted to oligomeric and monomeric short chain fatty acids, carbon dioxide or methane, and water (Liu et al., 2010, Roohi et al., 2018). Some depolymerases are polymer specific, while others degrade a multitude of products (Lucas et al., 2008). Polymer degradation rates are highly variable and influenced by the aforementioned abiotic factors, microbial populations and concentrations, as well as the physical characteristics of the polymer such as crystallinity, molecular weight, density or thickness, and surface area (Jendrossek and Handrick, 2002, Lucas et al., 2008, Su Yean et al., 2017, Fernandes et al., 2020).

Bacterial PHA depolymerases are found commonly from the *Cupriavidus*, *Alcaligenes*, *Comamonas*, and *Pseudomonas* genres (Vigneswari et al., 2015). *Pseudomonas* sp. are quite prevalent within ruminants, and along other cellulolytic bacteria are assumed to degrade PHA-based materials within the rumen (Bryant, 1959, Van Soest, 1994, Oyeleke and Okusanmi, 2008). Biodegradability of PBSA has been less widely evaluated, yet studies have demonstrated enzymatic degradation by *Roseateles depolymerans* (Shah et al., 2013), *Azospirillum* sp. (Wu, 2012), and *Leptothrix* sp. (Nakajima-Kambe et al., 2009), among others. No studies to date, however, have evaluated the ability of the ruminal microbiome to produce these extracellular depolymerases. Because most known bacteria that produce depolymerases are within the phylum Proteobacteria, which comprise one of the more abundant phyla present in the

rumen, ruminal microbes may have the ability to break down biodegradable polymer materials.

Ruminants are herbivorous animals with a unique digestive tract and digestion process to accommodate their forage-based diets. The rumen, one of four compartments within the stomach, serves as a fermentation vat where most nutrient absorption occurs (Van Soest, 1994). Ruminal fermentation is the process by which anaerobic and facultative microorganisms break down and ferment low-quality fibrous ingesta into utilizable energy sources for the animal, primarily volatile fatty acids (Castillo-González et al., 2013). The microbial consortium in the rumen consists of a wide diversity of organisms, with approximately 10^{11} cells/mL bacteria, 10^6 cells/mL protozoa, and 10^4 cells/mL fungi (Van Soest, 1994, Castillo-González et al., 2013, Owens and Basalan, 2016). This may foster an environment capable of degrading biodegradable polymers.

No prior research has specifically determined the ability of ruminants to degrade PHA-based materials and blends thereof within their gastrointestinal tracts. Estimating “true” digestive rates and the time needed to achieve maximum degradation of PHA materials and blends within the rumen is difficult due to the paucity of relevant literature and the large number of abiotic and biotic factors that influences those rates. However, it is necessary to assess if these materials are a safer alternative than polyethylene-based materials in netting applications if ingested by animals. The objective of this research was to assess digestibility kinetics of a proprietary PHA-based polymer and blends with PBSA via in vitro fermentation in rumen fluid. We hypothesized that biodegradable polymer materials would degrade in the ruminal environment, and that a blended material composed of PHA and PBSA would degrade at a faster rate than either material alone.

3. Materials and Methods

3.1. Animal Care and Use

The Institutional Animal Care and Use Committee of Virginia Tech approved all procedures involving dairy cows for collecting rumen contents (IACUC #18-229).

3.2. Sample Preparation for In Vitro Disappearance

Proprietary PHA-based polymer (Mirel P1004) nurdles produced by Metabolix, Inc. (Woburn, MA) were purchased from Alterra Plastics (Clifton, NJ). Poly(butylene succinate-*co*-adipate) (BioPBSTM) nurdles were purchased from Mitsubishi Chemical Performance Polymers (Greer, SC). A poly(butylene succinate-*co*-adipate) (PBSA; Bionelle 3001 MD; Showa Denko; Tokyo, Japan) and PHA (Mirel P1004) melt blend (90%wt PBSA, 10%wt PHA) in nurdle form as well as in a filament extrusion was developed. Briefly, PHA (Mirel P1004) and PBSA (Bionolle 3001MD; Showa Denko; Tokyo, Japan) were melt-blended and extruded using a pilot scale extruder at Alterra Plastics (Clifton, NJ). A filament was developed from this blend at North Carolina State University.

To test the effect of filtration bag type on in vitro disappearance, PHA nurdle (PHA), poly(butylene succinate-*co*-adipate) nurdle (PBSA), PBSA:PHA melt blend nurdle (Blend), and PBSA:PHA melt blend filament (Filament) were inserted into porous F57 and R510 Dacron bags (25-micron and 50-micron porosity, respectively) using a polymer to bag surface ratio of approximately 10mg/cm². F57 bags were filled with 250mg of sample and R510 bags were filled with 500mg of sample. All bags were double sealed using an impulse heat sealer, ensuring the mass-to-area ratio was not reduced.

3.3. In Vitro Disappearance

In vitro disappearance of polymers was conducted following methods outlined by Ferreira and Mertens (2005). Polymer samples were fermented in Daisy^{II} rotating jar in vitro incubators (Ankom Technology, Macedon, NY). In vitro fermentations were conducted over 10 days using two incubators, each containing four fermentation jars. Duplicates of all polymer treatments in F57 and R510 ANKOM bags were fermented in a jar for each fermentation time point (0, 3, 6, 12, 24, 48, 96, and 240h). In addition, duplicates containing corn silage standard, alfalfa hay standard, and blank bags of each type were included in each jar such that there were eight jars each filled with 28 bags, in vitro media, reducing agent, and inoculum as described below to maintain ruminal microbes throughout the study.

In vitro media and reducing agent were prepared as previously described (Goering and Van Soest, 1970). Briefly, a 20L buffer solution was prepared from ammonium bicarbonate and sodium bicarbonate in deionized water; a 20L macro-mineral solution was prepared from sodium phosphate anhydrous, potassium phosphate anhydrous, and magnesium sulfate pentahydrate in deionized water; and a 100mL micro-mineral solution was prepared from calcium chloride dihydrate, manganese chloride tetrahydrate, cobalt chloride hexahydrate, and ferrous chloride hexahydrate in deionized water. In vitro media (13.6L) was prepared by mixing trypticase, deionized water, micro-mineral solution, buffer solution, macro-mineral solution, and resazurin (0.1% w/v), in that order. Reducing agent (735mL) was prepared by mixing cysteine hydrochloride, deionized water, 1N sodium hydroxide, and sodium sulfide, in that order.

On the day of fermentation, the prepared bags were placed in their respective fermentation jars with 1200mL of media and warmed in a water bath at 39°C under continuous purging with carbon dioxide to maintain the anaerobic environment of a rumen. A 4-L flask containing 3360mL of media for blending with inoculum was also placed in a water bath at 39°C and purged with carbon dioxide. Reducing solution was prepared, 60mL was added to each fermentation jar, and 168mL was added to the media for blending. A composited inoculum was prepared with rumen fluid and rumen solids collected from three cannulated, lactating Holstein cows.

The composited inoculum was prepared as follows. For each of the three cows, two 2-L thermos flasks were filled with approximately 1L of rumen fluid and the remaining space was filled with rumen solids. Once in the laboratory, the top layer of surface solids exposed to the air was discarded from each thermos. The remaining mix of rumen fluid and rumen solids was strained through one layer of cheesecloth into a flask. Then, 550mL of strained rumen fluid from each thermos was collected and strained through two layers of cheesecloth into the composite flask. From each thermos, approximately 280g of strained solids was collected, mixed with 560mL of preheated and reduced media, and then blended for 15 and 45 seconds at low and high speed, respectively (Waring blender HGB-300, Waring Commercial, New Hartford, CT). The resulting blend was strained through two layers of cheesecloth into the composite flask. Every step was done under constant purging with carbon dioxide. After adding 800 mL of the composite inoculum to each fermentation jar, the jars were sealed with lids and placed into their designated incubators held at 39°C. The average pH of inoculum was 7.06 ± 0.12 and the average temperature was $39.3 \pm 0.5^\circ\text{C}$.

After designated incubation times, jars were removed from the incubator. Bags were removed from the jars, rinsed by hand with ice water until water ran clear, and dried in a forced-air oven for 24h at 55°C. Once fully dried, bags were weighed. Bags containing residues were corrected for the mass changes of respective blank bags during fermentation within incubation time to account for ruminal solids captured within bags. In vitro disappearance (IVD) was calculated according to equation [1]:

$$\text{IVD (\%)} = \frac{\text{Initial Dry Matter (g)} - \text{Corrected Undigested Residue (g)}}{\text{Initial Dry Matter (g)}} \times 100 \quad [1]$$

Digestion kinetic parameters for polymers and controls within the ruminal environment were estimated using the NLIN procedure in SAS according to the predicted digestibility equation [2] (Ørskov and McDonald, 1979):

$$\text{IVD (\%)} = A + B \times (1 - e^{(-k \times T)}) \quad [2]$$

where T is the time of incubation (h), A is the pool of immediately degraded material (%) at T = 0, B is the pool of potentially available material between 0 and 240h (%), and k is the fractional rate of disappearance (%/h) of pool B. B was estimated to be (100 – C – A), where C is the pool of non-digested material (%). For polymer treatments, C was estimated to be 0% because maximum degradation was not achieved during the study.

3.4. Thermochemical Analysis

Thermochemical analyses were conducted on samples incubated for 0, 24, 96, and 240h in F57 bags. These time points were selected based on mass loss trends seen after analysis. Since bag type did not have an influence on mass loss, only samples incubated in F57 bags were selected for analysis. Thermogravimetric analysis (TGA) of fermented samples was conducted using a TA Instruments TGA Q50 (TA Instruments, New Castle, DE). All samples were tested using aluminum pans in a nitrogen atmosphere. Samples

were heated at 10°C/min from room temperature to a maximum temperature of 450°C. The onset thermal degradation temperatures (T_d) were defined as the temperature at which 5% of the initial mass was lost from each degradation curve.

Differential scanning calorimetry (DSC) of fermented samples was performed using a TA Instruments DSC Q20. All samples were tested using aluminum pans in a nitrogen atmosphere. Samples of approximately 7.5 to 10.0mg were first heated at 10°C/min, cooled at 5°C/min, and heated again using the heat/cool/heat method. Poly(butylene succinate-*co*-adipate) was heated to a max heat of 150°C and cooled to 20°C; all other treatments were heated to 190°C and cooled to 25°C. The melting temperatures (T_m) were determined from endothermic peaks in the first heating scan.

3.5. Statistical Analyses

All parameters were analyzed to first compare polymer compositions in nurdle form and then to compare the PBSA:PHA melt blend in nurdle and filament formation. All statistical analyses were completed using the MIXED procedure in SAS 9.4 (SAS Institute, Cary, NC) and least squared differences were adjusted by Tukey' method. Polymer mass loss was analyzed with fixed effects of bag type, treatment, time, and interactions and random residual error. Disappearance kinetics parameters were analyzed with the fixed effects of bag type, treatment, the interaction, and random residual error. Some polymer treatments had two peaks within the first heating scan during differential scanning calorimetry due to multiple components; the initial peak was used for multiple comparisons of T_m . The PBSA:PHA polymers had two degradation curves after thermogravimetric analysis was conducted; the first point of degradation was determined to be the PHA component and the second point of degradation was determined to be the

PBSA component. Polymer T_d and T_m were analyzed with fixed effects of treatment, time, and the interaction and random residual error. All data were evaluated with an alpha value of 0.05.

4. Results

4.1. *In Vitro Disappearance of Biodegradable Polymers*

Bag type did not affect mass loss of polymer nurdles ($P = 0.07$) nor the PBSA:PHA blend in nurdle and filament formation ($P = 0.95$). Mass loss increased over time for each polymer treatment as expected, though significant mass loss was generally not achieved until 96h of incubation and did not change thereafter ([Figure 2.1](#)). By 96h, PHA achieved $0.25 \pm 0.13\%$, PBSA $0.44 \pm 0.16\%$, Blend $0.66 \pm 0.20\%$, and Filament $0.72 \pm 0.33\%$ more mass loss than at 0h.

Polymer treatments did not differ from one another in nurdle form. Though, we observed a two-fold increase of mass loss in the Filament compared to the Blend ([Figure 2.1](#), $P < 0.0001$). On average after 240h of incubation, PHA had $0.53 \pm 0.13\%$, PBSA had $0.49 \pm 0.16\%$, Blend had $0.82 \pm 0.20\%$, and Filament had $1.68 \pm 0.33\%$ total mass loss.

4.2. *Degradation Kinetics*

Ruminal digestibility kinetics parameters for polymer treatments and forage controls are shown in [Table 2.1](#). The filament formation of PBSA:PHA had the greatest pool of immediately disappeared material, nearly five times that of its nurdle counterpart ($P = 0.02$). The A fraction did not differ among polymers in nurdle composition ($P = 0.50$). Rates of degradation did not differ among the polymers in nurdle composition ($P = 0.38$) or among the PBSA:PHA blend in nurdle and filament composition ($P = 0.12$). The

maximum rate of disappearance achieved was 0.0031%/h by the Blend, substantially less than what was achieved by the forage controls.

Predicted digestibility curves of polymers using the parameters in [Table 2.1.](#), along with alfalfa hay and corn silage controls, are shown in [Figure 2.2.](#) The alfalfa hay and corn silage controls demonstrated the full logarithmic curve typically seen with forages during ruminal degradation, reaching maximum degradation around 96h indicated by a plateau. The polymer treatments, however, were still in the exponential phase of degradation indicated by a linear trend. When projected to 500h of incubation, degradation was predicted to not exceed 2%. At this rate, it would take upwards of 30 months to approach 50% degradation of the Blend in the ruminal environment.

4.3. Thermochemical Analysis

The first T_m from the first heating scan of polymers after incubation for 0, 24, 96, and 240h in rumen fluid are shown in [Figure 2.3.](#) Melting temperatures from the first heating scan were not different from the second scan. Two T_m s were observed in PHA as well as the PBSA:PHA blend in both nurdle and filament form, confirming the presence of more than one component in these polymers. However, two peaks were not present for the Blend or Filament until 24h, and the Filament did not present a second peak at 96h. Only the first peak temperature achieved within the first heating scan are shown for simplicity and comparison over time due to fermentation.

For PHA, two melting temperatures were clearly observed throughout the incubation period with significant increase in temperature by 96h. When PBSA was added to the proprietary PHA-based polymer formulation, changes were observed in the Blend and Filament melting temperatures. For the Blend, two distinct T_m s were not

detected until after 96 and 240h while for the Filament the two T_m s were observed after 24 and 240h. No significant increase in T_m was observed for PBSA or Blend until 240h. The Filament T_m did not differ across incubation times.

Both the Blend and Filament presented two onset thermal degradation temperatures after 0, 24, 96, and 240h as well. For assessment, these two temperatures were separated into the PHA component ([Figure 2.4.A](#)) and PBSA component ([Figure 2.4.B](#)). Interestingly, polymer T_d had an opposite trend with incubation time. For the PHA components, the T_d for PHA significantly decreased for 0, 24, and 96h consecutively (269.6, 213.2, 144.6°C, respectively). The T_d associated with the PHA component within both the Blend and Filament polymers significantly decreased from 0 to 24h by $119.5 \pm 1.0^\circ\text{C}$ and $114.0 \pm 1.6^\circ\text{C}$, respectively. For the PBSA components, the T_d for PBSA significantly decreased from 0, 24, to 96h consecutively (360.5, 209.4, 199.6°C, respectively). The T_d associated with the PBSA component within both the Blend and Filament significantly decreased from 0 to 24h by $137.0 \pm 1.9^\circ\text{C}$ and $135.5 \pm 0.8^\circ\text{C}$, respectively.

5. Discussion

Polyethylene-based bale netting materials are often incorporated into the diets of cattle. These materials are indigestible and significantly hinder animal health and productivity. Other potential materials should be identified and studied to develop more sustainable bale netting that may be safer for cattle if ingested. Polyhydroxyalkanoates and PBSA are viable materials to investigate as they are biodegradable by several genera of bacteria (Liu et al., 2010, Roohi et al., 2018). Known bacteria that produce extracellular enzymes that hydrolyze these materials largely belong to the Proteobacteria

phylum, which are abundant in the rumen (Bryant, 1959, Van Soest, 1994, Oyeleke and Okusanmi, 2008, Nakajima-Kambe et al., 2009, Wu, 2012, Shah et al., 2013, Vigneswari et al., 2015). With the abundant microbial community, elevated temperature, slightly acidic pH, and consistent motility in the rumen, the ruminal environment is presumed to foster degradation of PHA-based materials and blends with PBSA.

This is the first known study to specifically evaluate the ability of a ruminal environment to break down a PHA-based polymer and PBSA. Proprietary PHA-based polymer nurdle, PBSA nurdle, and a melt blend of the two in nurdle and filament form underwent fermentation for up to 10 days in rumen fluid in Daisy^{II} Incubators. Mass loss of residues was determined and used to estimate ruminal digestive kinetics. Residues additionally underwent TGA and DSC to evaluate structural alterations to materials.

Previous literature has evaluated growth and health of monogastrics supplemented with PHA-based materials that indicated their degradation in the gastrointestinal tract. Specifically, aquaculture species supplemented with PHB, a particular PHA material composed of hydroxybutyrate monomers, had increased growth, decreased pH, and enrichment of the gut microbial community that indicated their degradation (De Schryver et al., 2010, Liu et al., 2010, Sui et al., 2012). However, neither the PHA-based material utilized in this study nor PBSA and their blends' mass loss and degradation kinetics in animal digestive tracts have been evaluated, and therefore close comparisons of degradation cannot be made at this time. Degradation studies of PHA polymers are mostly conducted with PHB and its copolymers in soil, compost, sewage sludge, or river water, which tend to be static environments with different microbial ecologies, temperature, and pH than the rumen environment. Degradation of biodegradable

polymers is highly influenced by these factors, as well as the size, shape, and composition of the materials tested.

Although abiotic and biotic factors differ between these studies and the present one, the literature supports trends observed in our study in which multi-polymer blends degrade more readily than individual polymers. In a mass loss study over 35 days where PHB and copolymers were buried in ground soil at 28°C, PHB homopolymer films had the slowest rate of degradation at 0.93mg/d and achieved 10% degradation by 7 days. The fastest degrading copolymer with 4-hydroxybutyrate had a 75% increase in degradation rate at 1.63mg/d and achieved 30% mass loss at 7 days (Volova et al., 2017). This extreme increase in degradation compared to the present study in which we observed only 0.53% degradation of our PHA nurdle and 0.82% degradation of the melt blend with PBSA in nurdle formation after 10 days is likely attributed to structural form. The soil study utilized film discs that were approximately 30mm in diameter and 0.035 to 0.045mm thick (Volova et al., 2017). Our study utilized commercial nurdles that were approximately 3mm in diameter, significantly reducing the surface area to mass ratio.

Polymer formation may have a greater effect on degradation than composition. In a mass loss study in coastal waters over 160 days, films of PHB and a copolymer with 3-hydroxyvalerate had 58% and 54% degradation whereas their pellet counterparts had 38% and 13% degradation, respectively (Volova et al., 2010). Additionally, the pellets had slower rates of degradation. Differences were explained by the pellets having a decreased surface area interface with the environment, slowing microbial attachment to the polymer surface and extending the lag phase of degradation (Volova et al., 2010). This supports the two-fold increase in mass loss we observed with the Filament treatment

compared to its nurdle counterpart in this study. Filament pieces were approximately 1mm wide and 0.13mm thick, allowing for an increased polymer/rumen fluid interface and bacterial attachment compared to the nurdles.

No studies have evaluated a PBSA:PHA blend for mass loss and degradation kinetics due to biodegradation, and generally studies with PBSA are limited. In one study, dog-bone shaped specimens of polybutylene succinate (PBS) and PBSA underwent biodegradation in soil and compost for 24 weeks (Puchalski et al., 2018). Samples were also subjected to artificial weathering performed in cycles of exposure to UV light, elevated temperature, and artificial rainfall for up to 1800 h. When incubated in compost, PBSA mass loss was not observed until 4wk with 15% mass loss and it took PBS 12 weeks to achieve that same level of degradation. By 24wk, nearly 100% degradation of PBSA was achieved and PBS only achieved 70%. In soil, biodegradation of PBSA and PBS was significantly less, and when subjected to artificial weathering, less than 1% mass loss was observed by 1800h for both samples (Puchalski et al., 2018). Together, these results indicate that copolymers of PBS degrade faster, and increased microbial concentrations accelerate degradation of these materials as they do PHAs. As we compare these degradation rates to PHA-based materials in similar conditions (Volova et al., 2017), PBSA degrades at a slower rate than PHA-based materials which has been supported by another study (Salomez et al., 2019). In our study we observed that PBSA and PHA-based materials degrade at similar rates in the rumen environment; however, our study lasted only 10 days whereas former studies were conducted over a period of months and show deviations in degradation patterns that we may not be able to observe yet.

Trends seen within the present study after only 240h support studies at longer time frames to capture additional polymer degradation kinetics in the rumen. Some studies have described biodegradable polymer degradation to have a lag phase requiring microbial films to develop on the surface of polymers followed by a two-phase curve of mass loss in which rapid degradation is preceded by slower degradation for upwards of 10d (Wen and Lu, 2012, Volova et al., 2017). At only 240h of incubation, polymers in this study may still be in the first phase in which biofilms are developing and extracellular enzymes are beginning to hydrolyze polymer chains of the polymer surface. Although initial slow degradation will cause alterations to mechanical properties of the polymers, it may not cause significant mass loss. This could explain our observed trends with T_d and T_m within only 24h and the minimal mass loss.

Melting temperature and onset thermal degradation temperature indicate structural integrity of polymers in the face of thermal challenge. Evaluating changes in these values over time indicates structural changes of polymers due to degradation. The observed increase in T_m indicates shortening of polymer chains and increasing crystallinity of polymers in this study likely caused by preferential depolymerase degradation of amorphous material (Iwata et al., 1999, Sudesh and Abe, 2010). Similarly, decreasing T_d indicates a reduction in the thermal stability of the polymers, which may be explained by selective depolymerization of lower molecular weight polymer molecules in the amorphous region by 24h (Anunciado et al., 2021).

Previous studies support these findings in that PHA-based materials and blends thereof of various compositions show decreased molecular weight, increased melting temperatures, and decreased onset thermal degradation temperatures after environmental

degradation by microbes (Weng et al., 2011, Volova et al., 2017). Studies with PBSA indicate similar trends of increasing melting temperature and decreasing onset thermal degradation temperature due to enzymatic hydrolysis (Salomez et al., 2019). However, the present study does not control for abiotic hydrolysis; shortening of polymer chains within 24h of incubation could be due to water-induced hydrolysis in the bulk of the polymer, as rumen fluid is mostly composed of water (Cho et al., 2001, Salomez et al., 2019, Meereboer et al., 2020).

We propose that the slow, linear trend of degradation observed in this study is due to the beginnings of material surface erosion by bacteria (Meereboer et al., 2020). While abiotic factors may contribute to fracturing and mechanical alterations of biodegradable polymers, only biotic factors will result in mass loss due to enzymatic hydrolysis and cleavage that reduces chains to low molecular weight oligomers, dimers, and monomers that are either released into the environment or taken up by the surrounding microbes (Lucas et al., 2008, Salomez et al., 2019, Meereboer et al., 2020). Only one study has looked at the ability of microbes sampled from ruminal fluid to degrade PHA-based materials (Budwill et al., 1996); no studies have been conducted with PBSA. Isolates of *Staphylococcus* sp. from rumen fluid collected from one mature cow were capable of degrading PHB as indicated by the development of clearing zones after plating serial dilutions of rumen fluid on PHB overlay plates (Budwill et al., 1996). Our study utilized a composited inoculum composed of rumen fluid collected from three mature, lactating dairy cows on the same diet and in the same housing conditions. Preliminary evidence from these two studies suggests some ruminants may contain microbes that can produce these depolymerases; however, a general conclusion that all ruminants can degrade all

biodegradable polymers cannot be made at this time. Though a “core” microbiome containing 30 abundant bacterial groups was found in 90% of samples across a range of ruminant species, diets, and geographical regions, variation in bacterial diversity and prevalence still largely exists among animals primarily due to diet and housing (Van Soest, 1994, Henderson et al., 2015). Thus, the presence and abundance of depolymerase-producing bacteria in the rumen may vary among ruminants. Further incubation research with a larger sample size of ruminants under different housing and dietary conditions is needed to determine if capability of intraruminal degradation of these materials is universal across ruminants.

Concurrently, *in vivo* studies must be conducted under these conditions to establish differences between *in vitro* and *in vivo* systems. It is possible that results seen in this study are undermined by the limitations of an *in vitro* system. Although the Daisy^{II} Incubator *in vitro* system is a widely accepted and reliable method for determining true ruminal digestibility of feedstuffs (Tassone et al., 2020), it is a batch system with slight rotation. The live rumen is a continuous culture system with constant motility via contractions. *In vivo*, mechanical stress on polymers may be increased and accelerate abiotic fragmentation. This would allow more active bacterial formation of biofilms on larger surface areas and promote more rapid degradation and mass loss.

6. Conclusions

Based on these preliminary findings, PHA-based and PBSA materials are degradable in the rumen and may be a viable option to replace typical polyethylene-based agricultural plastics. Within 24h of incubation, polymer chains were likely cleaved via abiotic and biotic factors to alter mechanical structures of polymers as supported by

thermochemical analyses. However, though polymer chains were shortening, mass loss was unsubstantial by 240h of incubation, and the proprietary PHA-based polymer had the slowest rate of degradation. Further ruminal degradation studies of biodegradable polymers for longer duration are warranted to elucidate full degradation kinetics and the relative contributions of water-induced and enzymatic hydrolysis to degradation. These studies should be conducted with a range of ruminant hosts fed different diets and in different housing conditions to determine the general ability of all ruminants to host depolymerase-producing microbes.

7. Tables

Table 2.1. Ruminant disappearance kinetics parameters of alfalfa hay and corn silage forage controls and proprietary polyhydroxyalkanoate-based polymer nurdle (PHA), poly(butylene succinate-*co*-adipate) nurdle (PBSA), PBSA:PHA melt blend nurdle (90%wt PBSA, 10%wt PHA) (Blend), and a PBSA:PHA melt blend (90%wt PBSA, 10%wt PHA) filament (Filament).

Parameter							³ Composition		⁴ Formation	
	Alfalfa	Corn	PHA	PBSA	Blend	Filament	SEM	P-value	SEM	P-value
¹ A, %	43.26	58.18	0.13	0.08	0.20	0.99	0.07	0.50	0.14	0.02
² k, %/h	12.27	4.93	2.00E-3	2.10E-3	2.10E-3	8.85E-4	6.17E-6	0.38	8.25E-6	0.12

¹A = pool of immediately degraded material at T = 0

²k = fractional rate of disappearance of the pool of potentially available material

³Comparison of PHA, PBSA, and Blend treatments

⁴Comparison of Blend and Filament treatments

Data are shown as least squared means of treatments incubated in both R510 and F57 bags in duplicate.

Different letter superscripts in a row indicate significant differences between polymers ($P < 0.05$). Forage controls were not included in statistical analysis as only polymer degradation was of interest. Bag type nor its interactions were significant for neither the nurdles or two formations of PBSA:PHA blend.

8. Figures

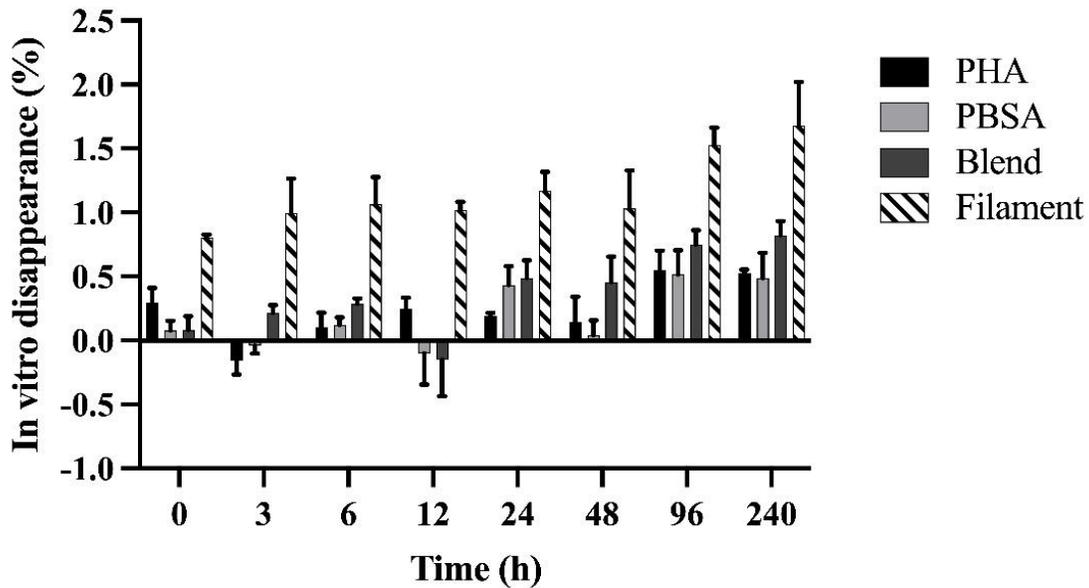


Figure 2.1. In vitro disappearance (%) of proprietary polyhydroxyalkanoate-based polymer nurdle (PHA), poly(butylene succinate-*co*-adipate) nurdle (PBSA), PBSA:PHA melt blend nurdle (90%wt PBSA, 10%wt PHA) (Blend), and a PBSA:PHA melt blend (90%wt PBSA, 10%wt PHA) filament (Filament) fermented in rumen fluid for up to 240h. When comparing nurdles, bag type and all its interactions, as well as the interaction of treatment and time, were not significant. Treatment: $P = 0.10$. Time: $P < 0.0001$. When comparing Blend and Filament treatments, bag type and all its interactions, as well as the interaction of treatment and time, were not significant. Treatment: $P < 0.0001$. Time: $P = 0.04$. Data shown are least squared means of polymers in F57 and R510 bags in duplicate (LSM \pm SEM).

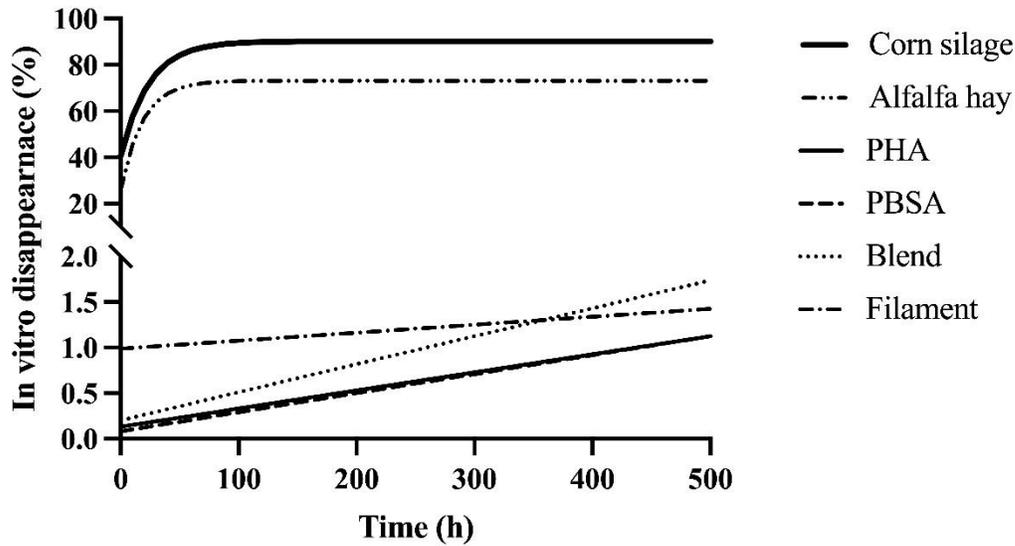


Figure 2.2. Projected degradation of forage controls alfalfa hay and corn silage and polymer treatments PHA, PBSA, Blend, and Filament. Projected degradation was determined from calculated disappearance kinetics parameters A and k as determined from the potential degradability equation ([Table 2.1](#)). Kinetics parameters plotted for polymer treatments are mean values of those fermented in F57 and R510 bags in duplicate. Kinetics parameters plotted for forage controls are mean values of those fermented in only F57 bags in duplicate as bag type was significant.

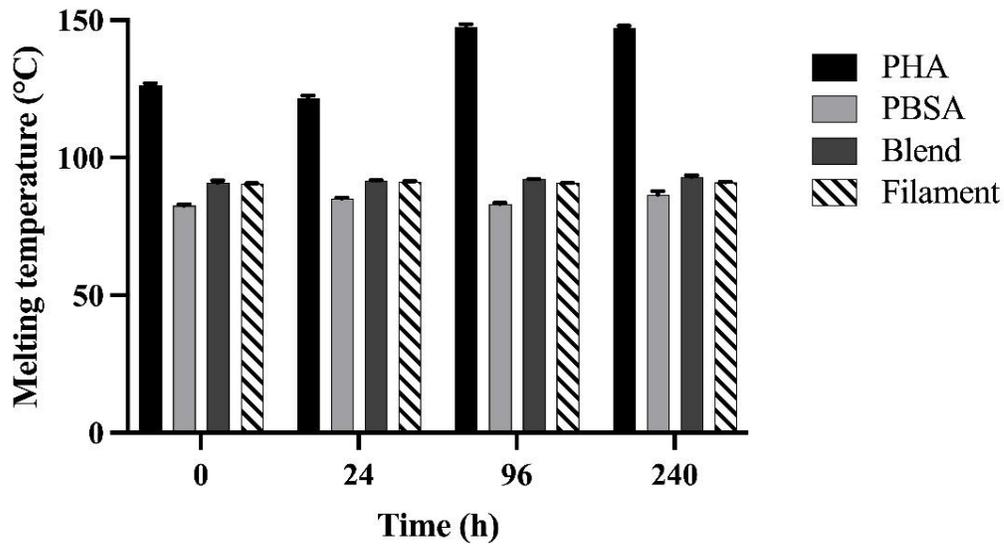


Figure 2.3. Melting temperature of PHA, PBSA, Blend, and Filament treatments after fermentation in rumen fluid for 0, 24, 96, and 240h in F57 bags. Melting temperature (°C) of treatments were determined from the first endothermic peak in the first heating scan. When comparing nurdles, Treatment: $P < 0.01$, Time: $P < 0.01$, Treatment x Time: $P < 0.01$. When comparing Blend and Filament treatments, Treatment: $P = 0.01$, Time: $P = 0.05$, Treatment x Time: $P = 0.23$. Data are shown as least squared means of treatments in duplicate (LSM \pm SEM).

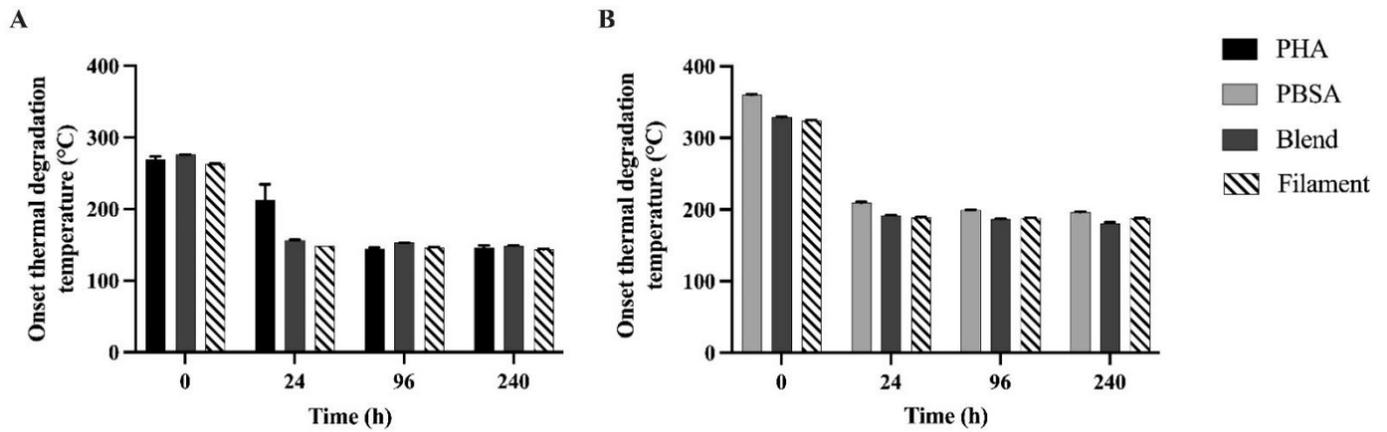


Figure 2.4. Onset degradation temperature of PHA, PBSA, Blend, and Filament treatments after fermentation in rumen fluid for 0, 24, 96, and 240h in F57 bags separated by: A) PHA component and B) PBSA component. Onset thermal degradation temperature (°C) of treatments were determined to be the temperature at which 5% of the initial mass degraded. For the PHA components, time was the only significant parameter. Time: $P < 0.0001$. For the PBSA components: Treatment: $P < 0.0001$, Time: $P < 0.0001$, Treatment x Time: $P < 0.0001$. Data are shown as least squared means of treatments in duplicate (LSM \pm SEM).

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CHAPTER 3

Long-term in situ ruminal degradation of polyhydroxyalkanoate, poly(butylene succinate-*co*-adipate), and low-density polyethylene in Holstein dairy cattle

1. Abstract

Using biodegradable materials such as polyhydroxyalkanoates (PHA) and poly(butylene succinate-*co*-adipate) (PBSA) to develop single-use agricultural plastics like bale netting may reduce the negative implications of plastic accumulation in the rumens of cattle. The objective of this research was to assess the long-term degradation of PHA, PBSA, and a PBSA:PHA blend (Blend) compared to a low-density polyethylene control (LDPE). Polyhydroxyalkanoates, PBSA, Blend, and LDPE films were incubated in the rumens of three cannulated, non-lactating Holsteins for 0, 1, 14, 30, 60, 90, 120, and 150d. In situ disappearance (ISD) and residue length were assessed after every incubation time. Data were analyzed with PROC MIXED in SAS and adjusted by Tukey's method to determine least squared differences between polymer treatments, incubation time, and the interaction. Polyhydroxyalkanoate achieved 100% degradation by 30d, with initiation occurring at 14d indicated by ISD and a reduction in residue length. The fractional rate of disappearance of PHA was 7.84%/d. Poly(butylene succinate-*co*-adipate) and Blend did not achieve any significant ISD, yet fragmentation of PBSA occurred at 60d and the Blend at just 1d likely due to abiotic hydrolysis. Low-density polyethylene achieved no ISD and residue length did not change over incubation time. We propose a PBSA:PHA blend is a valid alternative to polyethylene single-use agricultural plastic products based on its fragmentation within 1d of incubation. Future in vivo studies are warranted to

evaluate passage kinetics and potential influences on animal health with long-term feeding.

2. Introduction

Plastics may be incorporated into cattle rations by directly providing hay with netting still attached, or by grinding forage contaminated with residue plastic storage materials. These materials are indigestible and remain present within the rumen for the life of the animal. Approximately 20% of domestic cattle in abattoirs have foreign bodies within the rumen, of which 50 to 60% are plastic-based materials (Mushonga et al., 2015, Mekuanint et al., 2017). Long-term plastic presence and accumulation in the rumen reduces rumen functionality and may impact feed efficiency. Animals with plastic foreign bodies entrapped in the reticulorumen have decreased ruminal movement and microbial activity (Abd Al-Galil and Akraiem, 2016), erosion and ulcerations of ruminal papillae (Mekuanint et al., 2017, Otsyina et al., 2017, Harne et al., 2019), and increased heavy metal concentrations within rumen fluid (Mahadappa et al., 2020). Thus, it is imperative that a solution be found to reduce the incidence of plastic accumulation within the forestomachs of cattle. One approach is to develop net wrap from an alternative plastic material that is biodegradable and digestible by ruminants if it is ingested. Materials of interest include polyhydroxyalkanoate (PHA) and other biodegradable polymers such as poly(butylene succinate-*co*-adipate) (PBSA).

Polyhydroxyalkanoates are biodegradable polymers produced via fermentative processes within various microorganisms. This process occurs during unbalanced growth of microbes when select nutrient sources such as nitrogen are deprived, yet an abundant carbon source is available (Shen et al., 2009, Mudenur et al., 2019). Due to their

biodegradability in various environments, chemical diversity, insolubility in water, biocompatibility, and lack of toxins, they have a variety of applications including food-safe storage, agricultural mulch films, and medical supplies (Kadouri et al., 2005, Roohi et al., 2018, Amelia et al., 2019, Mudenur et al., 2019). However, PHAs are quite expensive and tend to be mechanically brittle. Blending PHAs with other biodegradable polymers, such as PBSA, improves its mechanical properties, biodegradability, and production costs which make them a more promising plastic alternative (Twarowska-Schmidt and Tomaszewski, 2008, Shen et al., 2009, Jompang et al., 2013).

We hypothesize that the rumen environment may foster the degradation of these plastic alternative materials. Biodegradable polymers such as PHAs and PBSA degrade by a combination of abiotic and biotic factors. Mechanical stress and increased temperatures may initiate the biodegradation process by inducing fracturing of the polymers, but these factors cause minimal mass loss alone (Lucas et al., 2008, Salomez et al., 2019). Mass loss of polymers is largely due to microbial production of extracellular depolymerases that cleave polymer chains, which are then assimilated into short chain fatty acids such as hydroxybutyrate, as well as carbon dioxide or methane and water (Liu et al., 2010, Roohi et al., 2018). Many bacteria that are known to produce extracellular depolymerases that degrade PHAs and PBSA are Proteobacterium (Nakajima-Kambe et al., 2009, Wu, 2012, Shah et al., 2013, Vigneswari et al., 2015). As Proteobacterium is one of the more abundant phyla present within the rumen (Bryant, 1959, Van Soest, 1994, Oyeleke and Okusanmi, 2008), ruminal microbes may have the ability to degrade and induce mass loss of biodegradable polymers.

We previously evaluated in vitro digestion kinetics of PHA, PBSA, and PBSA:PHA blend nurdles after fermentation in rumen fluid in Daisy^{II} Incubators over 240h. The results of this experiment indicated that the beginning stages of degradation may be occurring within 24h of fermentation in rumen fluid as indicated by increasing polymer melting temperatures and decreasing onset thermal degradation temperatures. However, mass loss was less than 1% for all polymers by 240h and the greatest fractional rate of disappearance rate was achieved by the PBSA:PHA blend at 0.0031%/h. Yet, results could have been undermined by the limitations of an in vitro system.

Studies evaluating forage digestion via in vitro and in situ methods in dairy cattle demonstrate that in vitro methods may underestimate degradability of various fibers (Gosselink et al., 2004, Bender et al., 2016). Additionally, the Daisy^{II} Incubator in vitro system is a batch system with slight rotation (Tassone et al., 2020). The live rumen is a continuous culture system with coordinated contractions to induce constant mixing of rumen contents (Van Soest, 1994). Thus, mechanical stress on polymers may be increased in the live rumen and cause abiotic fragmentation. This would allow more active bacterial formation of biofilms on larger surface areas and promote more rapid degradation and, potentially, mass loss. In situ studies for longer duration are warranted to evaluate true digestion kinetics of biodegradable polymers in the rumen environment to determine their suitability as plastic alternatives in the case of ingestion.

The objective of this research was to assess the long-term digestibility of a commercial PHA product and blends with PBSA compared to a polyethylene control via in situ fermentation in ruminally-cannulated Holstein cows over 5 months. We hypothesized that biodegradable polymer materials would degrade in the ruminal

environment while polyethylene would not, and that a blended material composed of a commercial PHA blend and PBSA would degrade at a faster rate than either material alone.

3. Materials and Methods

3.1 Animal Care and Use

The Institutional Animal Care and Use Committee of Virginia Tech approved all procedures involving dairy cows (IACUC #20-208).

3.2 Sample Preparation for In Situ Disappearance

Proprietary PHA-based polymer (Mirel P1004) nurdles produced by Metabolix, Inc. (Wo-burn, MA) were purchased from Alterra Plastics (Clifton, NJ). Poly(butylene succinate-*co*-adipate) (BioPBS™) nurdles were purchased from Mitsubishi Chemical Performance Polymers (Greer, SC). To develop a PBSA and PHA polymer blend (90% wt PBSA, 10% wt PHA), PHA (Mirel P1004) and PBSA (Bionolle 3001MD) were melt-blended and extruded into a nurdle formation using a pilot scale extruder at Alterra Plastics (Clifton, NJ). Low-density polyethylene (LDPE) was purchased from Sigma-Aldrich, Inc. (St. Louis, MO).

Films were produced from polymer nurdles using a melt press (#SP210C-X351220, PHI Hydraulics, Inc., City of Industry, CA). Approximately 100mg of nurdles were placed between two non-stick Kapton sheets on platens set to 174°C and incremental pressure to 340kg. The bottom platen was raised up near the top platen, and the polymer nurdles were given roughly 30s to soften before the bottom platen was raised the remaining way. The polymer nurdles were melted for approximately 30s to form films 0.1mm thick and 30 to 45mm in diameter.

3.3 In Situ Disappearance

For each of the polymer samples, 24 porous Dacron bags (R510, Ankom Technology, Macedon, NY) were filled with five polymer films (approximately 0.50g) and heat sealed. Bag dimensions were 5cm wide and 10cm long, and bag pore size was $50 \pm 10\mu\text{m}$. The sample amount to bag surface ratio was approximately $10\text{mg}/\text{cm}^2$. In addition to three empty bags that served as blank samples, three replicates of each of the four polymer samples were placed in each of eight 30.5cm by 38cm mesh laundry-type bags. At 1300h on the test day, all bags were simultaneously immersed within the rumens of three cannulated and non-lactating Holstein cows on pasture such that all cows received eight mesh bags each containing all treatments in triplicate. Bags were incubated for 0, 1, 14, 30, 60, 90, 120, and 150d. For the 0d incubation, bags were totally immersed into the liquid phase of the ventro-distal cavity of the rumen and extracted after 30s. Once the incubations were finished, bags were immediately removed and rinsed three or more times (3-min washing + spinning cycles) using a washing machine (SKY2767, Best Choice Products, Irvine, CA) until the rinse water ran clear. Residues were then dried in a forced-air oven at 55°C for 24h. Once fully dried, residues were weighed and corrected for the mass changes of respective blank bags during fermentation within incubation time and cow to account for ruminal solids not removed during the rinsing process. In situ disappearance (ISD) was calculated according to equation [1]:

$$\text{ISD (\%)} = \frac{\text{Initial Dry Matter (g)} - \text{Corrected Undigested Residue (g)}}{\text{Initial Dry Matter (g)}} \times 100. \quad [1]$$

Based on ISD of polymers, digestion kinetic parameters for PHA within the ruminal environment was estimated using the NLIN procedure in SAS according to the predicted digestibility equation [2] (Ørskov and McDonald, 1979):

$$\text{ISD (\%)} = A + B \times (1 - e^{(-k \times T)}), \quad [2]$$

where T is the time of incubation (h), A is the pool of immediately degraded material (%) at $T = 0$, B is the pool of potentially available material between 0 and 150d (%), and k is the fractional rate of disappearance (%/d) of pool B . B was estimated to be $(100 - C - A)$, where C is the pool of non-digested material (%). For this analysis, it was assumed that both A and C were equivalent to 0%, and B was equivalent to 100%.

After ISD was determined, three individual residue pieces were removed from each nylon bag. When degradation was partial and films were still distinguishable, three random film residues were selected and their length measured. When degradation became more complete and films were no longer distinguishable, three random residue fragments were selected and their length measured. Residue length was measured with a pair of digital calipers and the average length of polymer residues was determined.

3.4 Statistical Analysis

All statistical analyses were conducted with PROC MIXED and least squared differences were adjusted by Tukey' method in SAS 9.4 (SAS Institute, Cary, NC). Polymer ISD and residue length were both tested with the fixed effects of treatment, time, the interaction, and the random residual error, as well as the random effect of cow. Data were evaluated with an alpha value of 0.05.

4 Results

4.1 In Situ Disappearance of Polymers

In situ disappearance of polymers is demonstrated in [Figure 3.1](#). There was no ISD for any polymer after 1d of incubation in the rumen. Poly(butylene succinate-*co*-adipate), Blend, nor LDPE films achieved any significant ISD compared to 0d over the

entire incubation period. Films made of PHA showed exponential degradation. At 14d, ISD of PHA films increased by 55% compared to d0 ($P < 0.001$). From 14d to 30d there was an 80% increase in mass loss ($P < 0.001$), and PHA degraded by 100%. As ISD did not change across incubation time for the remaining polymers, their fractional rates of degradation were not determined. Based on the predicted digestibility equation, it was estimated that PHA's fractional rate of degradation was 7.84%/d. The predicted digestibility curve using this parameter is demonstrated in [Figure 3.2](#), and displays the typical logarithmic curve seen with forages.

4.2 Polymer Residue Length

Upon visual assessment of polymers ([Figure 3.3](#)), PHA, PBSA, and Blend films became embrittled and film residue size reduced over incubation time. Polymer residue length is demonstrated in [Figure 3.4](#). PHA film residue size decreased by 49% by 14d compared to 0d ($P < 0.0001$). Due to complete degradation of PHA films by 30d, length of PHA residues from 30d onwards is unavailable. Despite ISD not being significant for PBSA films until 120d, polymer residue length decreased by 29% by 60d compared to 0d ($P < 0.0001$). Poly(butylene succinate-*co*-adipate) films then decreased by an additional 7% to 6.84mm by 90d compared to 60d ($P < 0.0001$). Film residue length of PBSA did not decrease thereafter, though there was a tendency to decrease by another 7% by 150d compared to 90d ($P = 0.08$). Blend films began to fragment into smaller particles sooner than other polymers, decreasing by 29% by 1d compared to 0d ($P < 0.0001$). Blend films continued to fragment into smaller particles consecutively. Residue length decreased down to 13.30mm by 14d ($P < 0.0001$), and then down to 4.23mm by 30d ($P < 0.0001$).

Though residue length reduced to 0.65mm by 150d, length did not significantly change after 30d. LDPE films did not decrease in residue length throughout the study.

5 Discussion

Agricultural single-use plastics such as bale netting may be incorporated into cattle diets and negatively impact animal health and feed efficiency by disrupting ruminal epithelium integrity and the fermentative capability of microbes (Abd Al-Galil and Akraiem, 2016, Mekuanint et al., 2017, Otsyina et al., 2017, Harne et al., 2019, Mahadappa et al., 2020). With approximately 20% of domestic cattle containing plastic foreign bodies within the rumen, it is critical to resolve this issue. A possible solution is to develop a biodegradable and digestible net wrap from PHAs and PBSA.

This is the first known study to evaluate long-term digestion of biodegradable PHAs and PBSA in ruminants. Commercial PHA, PBSA, PBSA:PHA blend (90%wt PBSA, 10% wt PHA) and LDPE films underwent fermentation in three ruminally-cannulated Holstein cows for 150d. In situ disappearance was measured and used to determine ruminal digestion kinetics of polymers that actively degraded. Residues additionally were measured to determine decreases in polymer size in response to fermentation.

Our findings demonstrated that the ruminal environment was highly capable of degrading PHA and PBSA-based biodegradable polymers, likely by a combination of abiotic and biotic factors. Compared to the results of our in vitro study of fermentation of these same products, in situ degradation was increased. In vitro degradation of PHA resulted only in 0.53% mass loss by 10d with a fractional disappearance rate of

0.002%/h, whereas in situ degradation resulted in a remarkable 55% mass loss and a fractional degradation rate of 0.3%/h by 14d.

Possible explanations to this increased degradation in an in situ model include continuous inoculum and increased motility. Additionally, polymer formation utilized in this study compared to our in vitro model and the extended incubation period may have contributed to increased mass loss. Polymers in this study were pressed into films that were 0.1mm thick and approximately 35mm in diameter whereas our in vitro study evaluated polymers in their commercial nurdle formation that were approximately 3mm in diameter. A study of poly(3-hydroxybutyrate) (PHB) and a copolymer with poly(3-hydroxyvalerate) (PHV) biodegradation in tropical coastal waters over 160d found that films that were 0.1mm thick with a 30mm diameter had 53% and 315% increases in mass loss, respectively, compared to 10mm pellets of the same chemical structures (Volova et al., 2010). It was determined that polymer formation has a critical influence on degradation rates through mass to surface area ratio. Similar conclusions were made by a study with the same polymers in film and pellet formation in tropical soils over 10 months (Boyandin et al., 2013). Increased mass to surface area ratio likely allows a greater interface between the polymer surface and the microbial consortium within the rumen. This would enhance bacterial attachment and enzymatic hydrolysis via extracellular depolymerases. This could in part be responsible for the great increase of biodegradation in this study compared to our in vitro model.

Additionally, previous studies indicated there may be a lag phase of biodegradation of PHA-based materials, requiring time for bacterial attachment to polymer surfaces. Depending on the microbial population within the environment of

degradation and polymer composition, the lag or stationary phase can be anywhere from a few days to months (Imam et al., 1999, Volova et al., 2010, Wen and Lu, 2012). It is plausible that at 10d of incubation within rumen fluid polymers are still in the stationary phase in which little to no mass loss occurs. The lag phase could be overcome between 10 and 14d of incubation in rumen fluid, leading to the detectable exponential increase in in situ disappearance. Further studies in which in situ biodegradation of commercial PHA is more readily evaluated between 1 and 14d are needed to more accurately detect if there are two periods of mass loss for proprietary PHA blend and if adjusted fractional rates of degradation are needed.

Though PHA digestibility was greatly improved, PBSA nor PBSA:PHA disappearance was improved by an in situ approach for longer durations. Despite the lack of mass loss, there was a significant decrease in polymer residue length of PBSA by 60d and of the Blend, composed of 90% PBSA, by just 1d of incubation in the rumens of dairy cattle. The comparing trends of in situ disappearance and polymer residue length indicate that synthetic biodegradable polymers such as PBSA and blends mostly comprised of them may degrade more by abiotic factors than biotic in the rumen environment.

Hydrolytic cleavage of polymer chains occurs when water molecules penetrate the amorphous region and act by random chain scission to reduce molecular weight and induce fragmentation. Through this process, intact polymers burst into smaller fragments, yet mass loss does not necessarily occur (Albertsson et al., 1994, Hakkarainen, 2001, Salomez et al., 2019). Enzymatic degradation of polymers initiates at a slower rate and hydrolyzes polymer chains at the surface level due to enzymes being too large to enter

the amorphous region of the polymer (Hakkarainen, 2001, Lucas et al., 2008).

Oligomeric and monomeric constituents are released, and mass loss of the polymer is detected. Thus, we propose that fragmentation of PBSA and the Blend is largely induced by spontaneous water hydrolysis due to polymer submersion in rumen fluid, while PHA biodegradation is largely through enzymatic hydrolysis by PHA depolymerases produced by the microbial consortium. This is also supported by visual appraisal of polymer residues in which the 14d residues of PHA were porous with organic degradation of the outermost edges, while PBSA at 60d and PBSA at 1d had brittle residues with sharp geometric edges and no visible appearance of surface erosion. Scanning electron microscope images of polymer surfaces after incubation are needed to confirm if surface erosion is present.

Comparing PBSA by itself to the Blend, polymer fragmentation occurred at a much slower rate despite the mechanism of degradation likely being the same. It is probable that the improved fragmentation is due to the inclusion of PHA. No known studies have evaluated the differences in biodegradation of a PBSA and PHA blend in any environment. However, a study in which polybutylene succinate (PBS) and PBSA underwent biodegradation in compost found that PBS took 6 weeks to begin to fragment, while more complex PBSA began to fragment at just 4 weeks (Puchalski et al., 2018). The trend that more complex aliphatic polymers degrade more readily than their constituents hold true throughout the literature (Mergaert et al., 1993, Sridewi et al., 2006, Kupczak et al., 2021). This is largely due to decreased molecular weight induced by chain scission during the development process and improved hydrophilicity.

A PBSA:PHA blend appears to be a promising alternative material to typical polyethylene that is often used for single-use agricultural plastics such as bale netting in the case of animal ingestion due its improved mechanical properties compared to PHA and degradability. Though ISD was not significant, polymer residue size began decreasing within 24h of incubation. Polyethylene products negatively impact animal health and productivity due to their indigestibility and residency time within the rumen as they cannot escape the rumen to the rest of the digestive tract due to size (Pizol et al., 2017). Our results support the inability of the ruminal environment to degrade polyethylene products as LDPE did not achieve significant ISD nor a reduction in residue length over 150d of incubation. Based on our findings, we suggest that bale netting developed from a PBSA:PHA blend will not cause the same inflictions as polyethylene material if consumed due to the probability of the material passing through the rest of the gastrointestinal tract due to degradation in the rumen. Previous studies indicate that particles less than 1.18mm are more readily able to escape the ruminoreticulum to the omasum (Poppi et al., 1981, Schulze et al., 2014, Sutherland, 2019). Thus, it is concluded that a PBSA:PHA blend should be able to escape the ruminoreticulum by 150d, if not before. However, in vivo studies in which PBSA:PHA is fed to cattle are warranted to elucidate not only the ability of the polymer to pass through the digestive tract, but to evaluate any potential influences of long-term consumption of the material and its potential degradation within the rest of the gastrointestinal tract.

6 Conclusions

Based on our findings, PHA-based and PBSA materials are degradable in the rumen while polyethylene-based materials are not. A PBSA:PHA material may be a

viable option to replace typical polyethylene-based agricultural plastics in the case of animal ingestion. Although ISD did not occur, within 1d of incubation PBSA:PHA polymers began to fragment. Reducing polymer size within the rumen may allow passage through the reticulorumen and reduce incidences of plastic impaction. To determine the exact mechanisms behind PHA and PBSA degradation in the rumen environment, further in vitro and in situ studies are needed to evaluate enzymatic hydrolysis, surface erosion, and degradation products. Future in vivo studies are also necessary to elucidate the ability of biodegradable polymers to pass through the digestive tract and the subsequent health of animals with long-term consumption of the material.

7 Figures

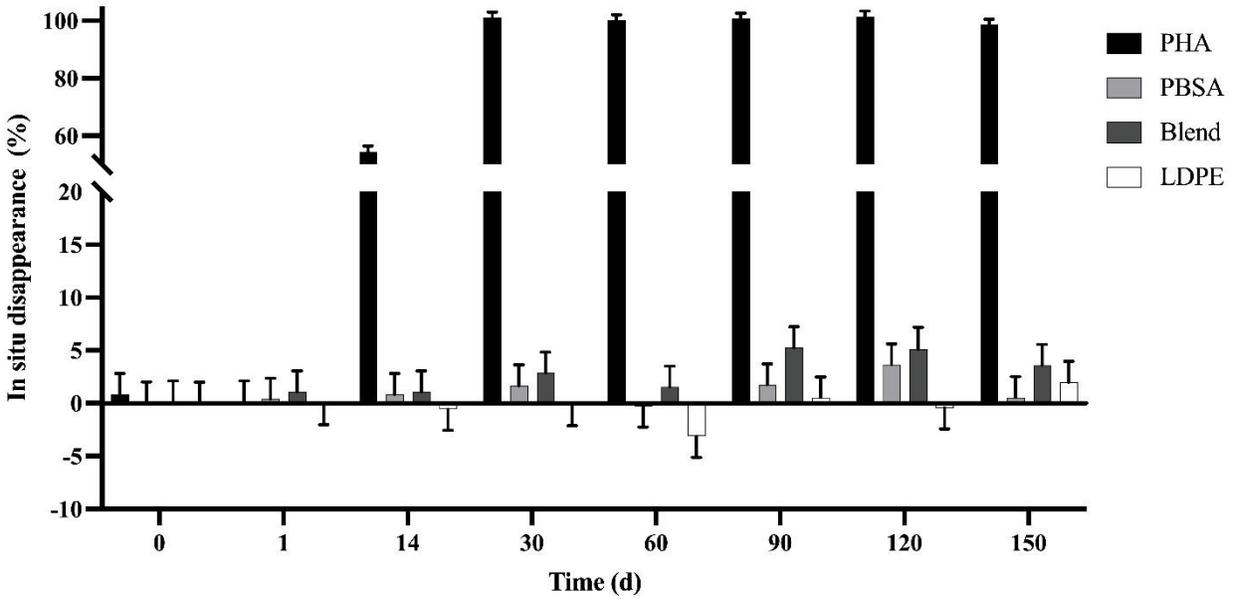


Figure 3.1. In situ disappearance (%) of commercial polyhydroxyalkanoate blend (PHA), poly(butylene succinate-*co*-adipate) (PBSA), PBSA:PHA (90% wt PBSA, 10% wt PHA) (Blend), and low-density polyethylene (LDPE) films incubated in the rumens of three cannulated, non-lactating Holsteins for up to 150d. Treatment: $P < 0.001$. Time: $P < 0.001$. Treatment x Time: $P < 0.001$. Data are shown as least squared means of nine replicates distributed among three cows (LSM \pm SEM).

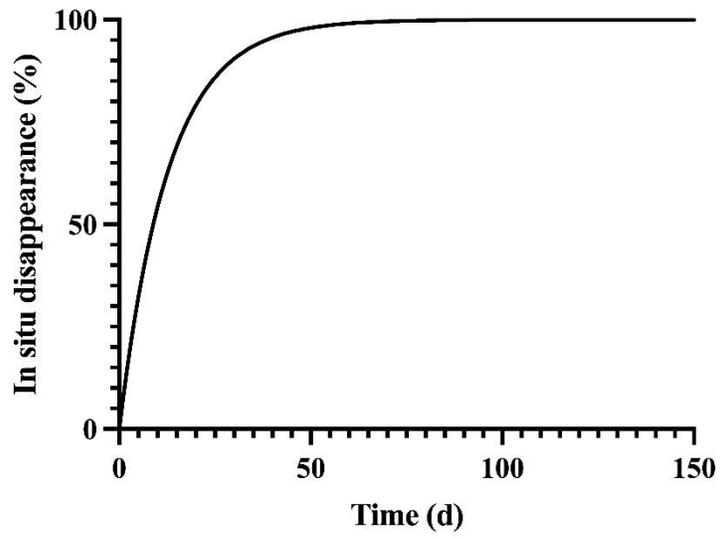


Figure 3.2. Degradation of PHA modeled by the predicted digestibility equation.

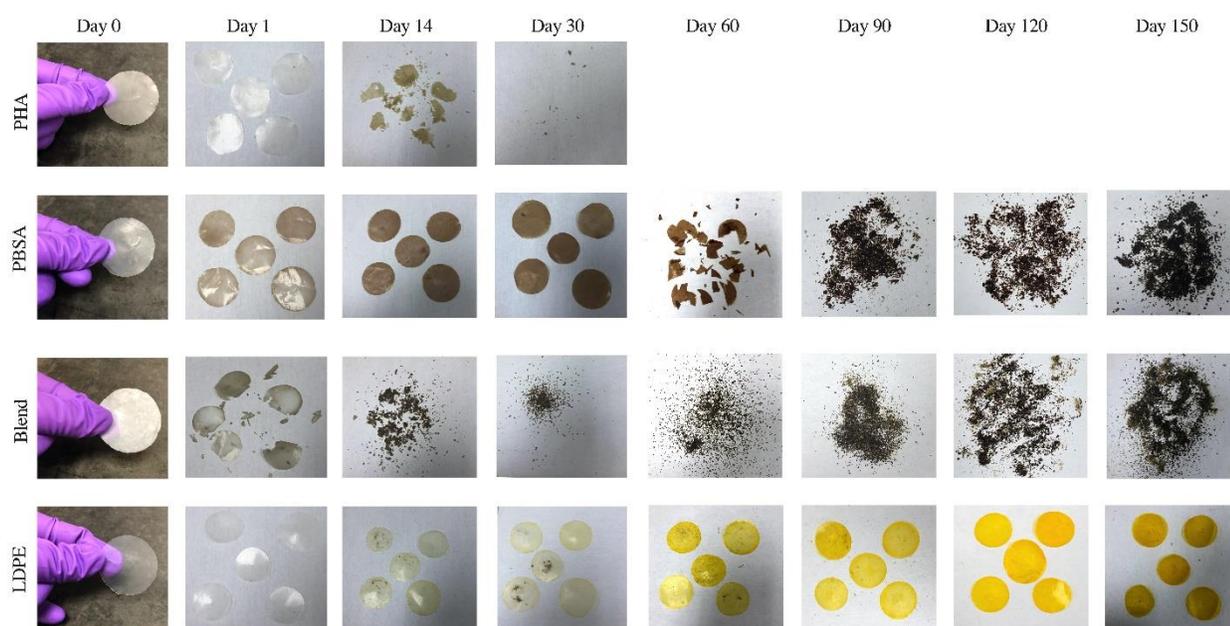


Figure 3.3. Visual assessment of PHA, PBSA, Blend, and LDPE films incubated in the rumens of three cannulated, non-lactating Holstein cows for up to 150d.

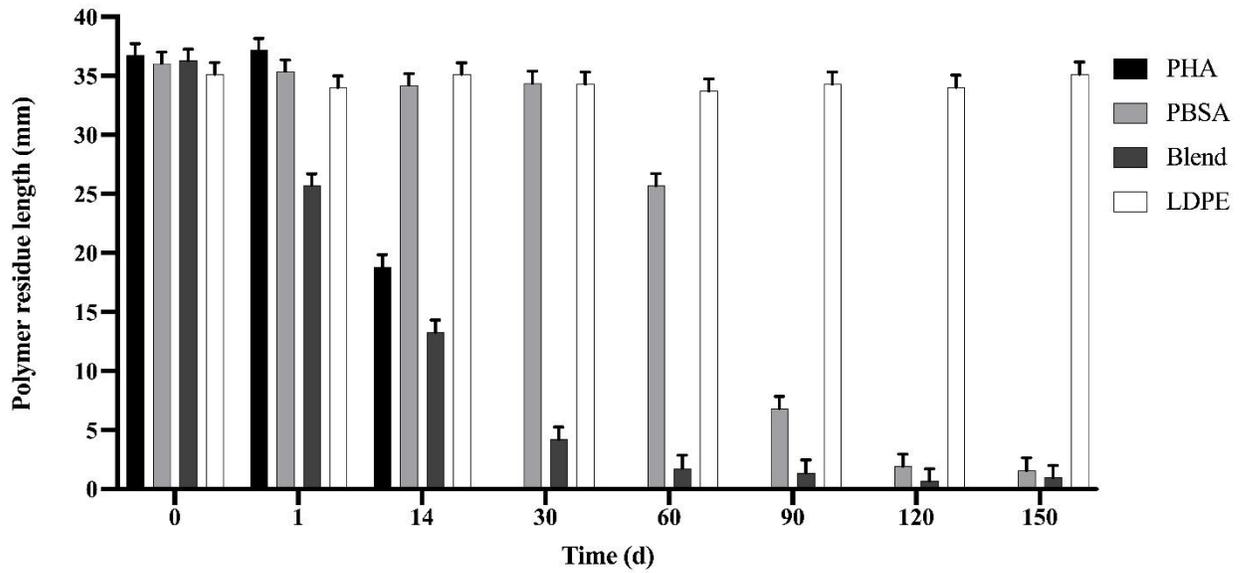


Figure 3.4. Residue length (mm) of PHA, PBSA, Blend, and LDPE films incubated in the rumens of three cannulated, non-lactating Holstein cows for up to 150d. Treatment: $P < 0.0001$. Time: $P < 0.0001$. Treatment x Time: $P < 0.0001$. Data are shown as least squared means of nine replicates distributed among three cows (LSM \pm SEM).

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CHAPTER 4

Clearance of poly(butylene succinate-*co*-adipate):polyhydroxyalkanoate melt-blend and low-density polyethylene films from the rumens of Holstein bull calves

1. Abstract

Due to the occurrence of plastic impaction in ruminants and its deleterious influences on health and production, it is necessary to determine the suitability of biodegradable polymers such as polyhydroxyalkanoates (PHA) and poly(butylene succinate-*co*-adipate) (PBSA) to replace low-density polyethylene-based single-use agricultural plastics. The objectives of this study were to evaluate the clearance of a PBSA:PHA melt-blend polymer compared to low-density polyethylene (LDPE) and to evaluate animal health when these materials are fed to cattle. Twelve Holstein bull calves were blocked by age and weight and randomly allocated to one of three daily bolus treatments: 13.6g of PBSA:PHA in 4 gelatin capsules (Blend), 13.6g of LDPE in 4 gelatin capsules (LDPE), or 4 empty gelatin capsules (Control) for 30d. Hemograms were conducted on blood samples collected on d0 and d30. On d31, animals were sacrificed to evaluate gross rumen measurements and pathology, determine papillae length, and characterize polymer residues present in rumen contents. Data were analyzed with PROC MIXED in SAS and adjusted by Tukey's method to determine least squared differences between treatments. Feed intake, body weight, body temperature, and general health were determined throughout the study. No animals presented any symptoms related to plastic impaction and animal health was not affected by treatment. Daily grain and hay intake, body weight, rectal temperature, hematological parameters, gross rumen measurements and pathology, and rumen pH and temperature were not affected by treatments. Methylene

blue reduction time of Blend calves tended to decrease by 30% compared to LDPE calves, and caudal ventral papillae length of Blend calves was 50% longer than those of Control animals. This could potentially be due to subsequent effects of biodegradation products, such as butyrate, though studies are needed to specifically elucidate this. Despite health not being influenced by either PBSA:PHA or LDPE polymers, polymer accumulation and residue length differed among treatments. Calves dosed with LDPE had 27.42g of undegraded polymer retained in the rumen while Blend calves had only 1.72g of fragmented polymers that were 10% of their original size. Single-use agricultural plastics developed from PBSA:PHA may be a suitable alternative to LDPE-based products in the case of ingestion in ruminants due to fragmentation of polymers and improved clearance from the reticulorumen. As such, utilization of these materials may reduce the incidence of plastic impaction in ruminants in commercial operations.

2. Introduction

Plastic impaction is detrimental to ruminant health and should be a concern for the dairy industry as it negatively impacts animal health and productivity. Approximately 20% of cattle are estimated to be afflicted by plastic impaction (Mushonga et al., 2015, Mekuanint et al., 2017). Plastic impaction occurs when large amounts of indigestible plastic materials are indiscriminately ingested by ruminants in either a single meal or over the lifetime of the animal. These materials may be introduced into cattle rations by chopping forages with intact netting. In this condition, it is estimated that 0.07% of the diet contains polyethylene-based plastics (Pizol et al., 2017). When these materials enter the reticulorumen, they aggregate together to form large ball-like masses that press against rumen epithelium during rumination (Hailat et al., 1998, Bakhiet, 2008, Otsyina

et al., 2017a, Pizol et al., 2017, Martín Martel et al., 2021). With long-term presence, plastics reduce rumen functionality and may impact feed efficiency by decreasing ruminal movement and methylene blue reduction time, an indirect measure of microbial fermentation, (Abd Al-Galil and Akraiem, 2016) and inducing pathological alterations to epithelium (Mekuanint et al., 2017, Otsyina et al., 2017a, Harne et al., 2019). To reduce the incidence of plastic impaction, it is crucial to identify biodegradable alternatives to polyethylene that will safely degrade within the rumen environment with no afflictions on animal health. Potential materials include polyhydroxyalkanoates (PHA) and other biodegradable polymers such as poly(butylene succinate-*co*-adipate) (PBSA).

We previously evaluated in situ ruminal degradation of PHA, PBSA, PBSA:PHA blend, and low-density polyethylene (LDPE) polymer films in dairy cattle for 150d (Galyon et al., *in progress*). The results of this experiment confirmed that polyethylene materials do not degrade within the rumen, and that a PBSA:PHA blend may be the best material to replace LDPE for net wrap applications. Although mass loss did not occur until 120d, the PBSA:PHA blend in our in situ study began to fragment to 29% of their original size by just 24h of inclusion in the rumen (Galyon et al., *in progress*). Thus, this polymer blend may fragment to small enough pieces to pass through the reticulo-omasal orifice and through the rest of the digestive tract, decreasing the incidence of plastic accumulation within the rumen. Additionally, the inclusion of PBSA with PHA improves its mechanical properties, biodegradability, and production costs, making a blend an attractive option over PHA by itself (Mushonga et al., 2015, Mekuanint et al., 2017). Before development of plastic net wraps from a PBSA:PHA material can be considered,

in vivo studies in which the clearance of PBSA:PHA from the reticulorumen after feeding and subsequent influences on animal health are necessary.

The objectives of this study were to: (1) evaluate the clearance of a PBSA:PHA melt-blend polymer compared to LDPE, and (2) evaluate animal health when these materials are fed to cattle. We hypothesized that if PBSA:PHA was fed to cattle that it would fragment and largely pass through the reticulorumen, and that health parameters would not differ from healthy control animals. If LDPE was fed however, it was hypothesized that the material would not degrade, would accumulate within the reticulorumen, and negatively impact animal health.

3. Materials and methods

3.1. Treatment Preparation

A poly(butylene succinate-*co*-adipate) (PBSA) and polyhydroxyalkanoate (PHA) polymer blend (90% wt PBSA, 10% wt PHA) was developed by melt-blending and extruding a proprietary PHA-based polymer (Mirel P1004, Metabolix, Inc., Woburn, MA) and PBSA (Bionolle 3001MD, Showa Denko America, Inc., New York, NY) into a nurdle formation using a pilot-scale extruder at Alterra Plastics (Clifton, NJ). Low-density polyethylene (LDPE) nurdles were purchased from Sigma-Aldrich, Inc. (St. Louis, MO). Films were produced from polymer nurdles using a melt press (#SP210C-X351220, PHI Hydraulics, Inc., City of Industry, CA). Nurdles were placed between two non-stick Kapton sheets on platens set to 174°C and incremental pressure to 340kg. The bottom platen was raised near the top platen, and the polymer nurdles were given roughly 30 s to soften before the bottom platen was raised entirely. The polymer nurdles were melted for approximately 30s to form circular films 0.1mm thick. Circular films were

then run through a cross-cutting paper shredder (#ITSH-555, Innovative Technology Americas, Inc., Pewaukee, WI) with 4mm blades to produce PBSA:PHA and LDPE films that were 50.4 ± 19.1 mm long.

To prepare daily boluses for treatments, 3.4g of PBSA:PHA or LDPE films were packed in 3/8oz Torpac gelatin capsules (Fairfield, NJ). Three daily treatments were utilized in this study: 4 empty gelatin capsules (Control), 13.6g of PBSA:PHA in 4 gelatin capsules (Blend), or 13.6g of LDPE in 4 gelatin capsules (LDPE).

3.2. Calf Health and Management

The Animal Care and Use Committee of Virginia Tech approved all animal procedures (IACUC #19-265). In this 6-wk study, 12 weaned Holstein bull calves were purchased from a single farm (Floyd, VA) and transported to Virginia Tech (Blacksburg, VA) on January 10, 2022. Upon arrival, calves were evenly blocked into 4 groups by age (62 ± 9 d) and initial body weight (74.9 ± 8.0 kg). Calves were individually housed in tie-stall pens (122cm x 183cm) in temperature (20°C) and light-controlled rooms (12h of light) for the duration of the study. Pens were placed on top of rubber mats (122cm x 183cm) and pine shavings were provided for bedding. Calves had visual and auditory contact with each other, as well as nose-to-nose contact between adjacent stalls.

Calves were acclimated for 14 to 15d before the start of the study depending on the start of treatment administration as described below. Upon arrival, calves were offered 2.3kg of calf starter (Producer's Pride, Brentwood, TN), ad libitum mixed-grass hay, and ad libitum water. According to the manufacturer's specifications, the calf starter contained 16.2% crude protein, 1.6% crude fat, 13.0% crude fiber, and 17.0% acid detergent fiber. Calves were acclimated to 2.7kg of starter by d0 and continued to receive

ad libitum mixed-grass hay and water. Feeding occurred once daily at 0700h and refusals were weighed separately for starter and hay the next morning before feeding.

Calves were randomly assigned to treatments as a randomized complete block design such that 4 calves were assigned to Control, 4 calves were assigned to Blend, and 4 calves were assigned to LDPE treatments. Treatment administration occurred directly after feeding via a plastic bolus gun from d0 to d30. Two groups were randomly selected to start one day, and the remaining two groups started the next to ensure treatments were administered for 30d before tissue collection on two consecutive dates.

Calf health was assessed twice daily, at 0700h with feeding and again at 1500h. Rectal temperature, fecal score, obvious treatment presence in fecal matter, bloat occurrence, eye score, nasal score, and activity were evaluated. Fecal score was evaluated with a 5-point scale: 1 = watery, 2 = loose, 3 = semi-formed and pasty, 4 = hard pellets, 5 = normal. Eye score was evaluated with a 4-point scale: 1 = heavy ocular discharge, 2 = moderate bilateral discharge, 3 = limited discharge, and 4 = no discharge. Nasal score was evaluated with a 4-point scale: 1 = copious amounts of mucus discharge, 2 = bilateral cloudy mucus discharge, 3 = small amount of unilateral discharge, and 4 = no discharge. It was noted if a calf exhibited a fecal, nasal, or eye score equal to or below 2, lethargy with depressed ears, a cough or elevated respiration rate, a rectal temp above 40.0°C, or reduced feed or water intake. A veterinarian was sought if any of these symptoms presented for more than a day or if two or more of these symptoms occurred simultaneously. Every week, calves were weighed by walking onto scales, and average rectal temperature, daily grain intake, and daily hay intake were determined.

3.3.Hematological Evaluation

Whole blood samples were collected from animals via venipuncture of the jugular vein into 7ml vacutainers containing ethylenediaminetetraacetic acid (EDTA) on d0 and d30 of bolus administration. Within an hour of collection, approximately 1ml of mixed blood with EDTA was taken up by syringe for determination of erythrocyte sedimentation rate (ESR) using the Wintrobe tube method to determine potential amatory activity. Blood was deposited into perpendicular Wintrobe tubes to the zero mark. After 24h, the fall of red-packed cells was measured in millimeters.

The remaining blood samples were delivered to the Virginia Tech Animal Laboratory Services (VITALS) within 2h of collection for large animal hemogram analysis via a Sysmex XN-1000 (Sysmex America, Inc., Lincolnshire, IL). This analysis includes the determination of red blood cells (RBC), hemoglobin (HB), hematocrit, mean corpuscular volume (MCV), mean corpuscular hemoglobin concentration (MCHC), red cell distribution width (RDW), reticulocytes, white blood cells (WBC), neutrophils, monocytes, eosinophils, basophils, platelets, and mean platelet volume (MPV).

3.4 Tissue Collection and Gross Rumen Measurements

Calves were slaughtered via captive-bolt stunning followed by exsanguination on d31 between 0800h and 1100h, at least 2h after feed was removed. The entire gastrointestinal tract was ligated and removed from the carcass. The full forestomach was removed, weighed in full, and then segmented at the reticulo-omasal orifice. The reticulorumen was then weighed full before being opened to measure pH and temperature immediately. A small sample of total contents was strained by hand to retrieve approximately 10ml of rumen fluid to determine methylene blue reduction time (MBRT). Methylene blue reduction time was determined as the time for the blue coloration to

disappear from rumen fluid after 0.5ml of 0.04% methylene blue solution was added to the rumen fluid (Petrovski, 2017). Total rumen contents were then thoroughly mixed by hand and a small sample was collected. The total weight of the sample was determined, and then the rumen solids were strained through two layers of cheesecloth and reweighed. Solids were flash-frozen until further analysis to determine the amount and size of polymer residues present. All solids were evacuated from the reticulorumen and then subjectively scored using a 5-point scale: 0 = no polymer present, 1 = small amount of degraded polymer present, 2 = degraded polymer present throughout contents, 3 = small amount of intact polymer present, and 4 = intact polymer present throughout contents.

The empty reticulorumen was weighed to determine the total rumen contents and then rinsed to remove any remaining particles loosely attached. The reticulorumen was “butterfly” cut for a full visual of the epithelium. The epithelium was grossly evaluated in the cranial dorsal, caudal dorsal, cranial ventral, and caudal dorsal regions using a 6-point scoring system following Jonsson et al. (2020): 0 = no evidence of any damage, 1 = palm-size or smaller areas bare of papillae, 2 = large areas bare of papillae, 3 = areas of scarring, 4 = red/bloody areas, 5 = areas of parakeratosis. Rumen epithelium samples for papillae measurements were obtained from the cranial dorsal and caudal ventral regions using a scalpel and a 3cm x 10cm stencil. Epithelium samples were stapled to wooden tongue depressors and immediately placed in 10% formalin to fix for 48h.

3.5 Rumen Histological Measurement

Epithelium sections of 0.5cm were cut from fixed tissue samples, placed in cassettes in 10% formalin, and sent to VITALS for routine processing. This included embedding in paraffin, sectioning 5- μ m thick sections and mounting onto 3 positively

charged microscope slides, and hematoxylin and eosin staining following standard procedure. Digital images of slides were taken at 4x using an Olympus BX43 microscope (Olympus Corporation of the Americas, Center Valley, PA) fitted with a Retiga R6 camera (QImaging Corporation, Surrey, BC, Canada). Images were viewed in ImageJ (U.S. National Institutes of Health, Bethesda, MD) for measurement of papillae length using the line tool. Three random papillae from each slide were selected and measured.

3.6. *Polymer Residues*

Frozen rumen solids samples were thawed to room temperature and then dried in a forced-air oven at 55°C for 48h. Total dried sample weight was determined and polymer particles were manually removed with forceps. Residual polymers were weighed in total and then three random particles were selected, and their length was measured with digital calipers. Total residual polymer present within the rumen was estimated using the following:

$$\text{Accumulation (g)} = \text{contents (g)} \times \text{solids (\%)} \times \text{DM (\%)} \times \text{polymer (\%)},$$

where *contents* indicates the total weight of all rumen contents, *solids* indicates the ratio of solids to total weight of rumen contents, *DM* indicates the dry matter content of solids, and *polymer* indicates the ratio of polymer to dry solids weight.

3.7. *Statistical Analysis*

Unless otherwise mentioned, all data were analyzed using the MIXED procedure and least squared differences were adjusted by Tukey's method in SAS 9.4 (SAS Institute, Cary, NC). To improve the normality of the data, average daily grain intake was transformed by the power of 5, and average daily hay intake was transformed by taking the square root for analysis. Average daily grain intake, average daily hay intake, body

weight, and rectal temperature were analyzed with the repeated measure of week, the fixed effects of treatment, week, and the interaction, and the random effects of animal and group. Hematological parameters were analyzed with the repeated measure of sampling day, the fixed effects of treatment, sampling day, and the interaction, and the random effects of animal and group. Subjective scoring parameters of rumen contents and gross pathology of epithelium regions were analyzed using PROC FREQ and the fixed effect of treatment. Papillae length was analyzed with the fixed effects of treatment, region, and the interaction, and the random effects of animal, group, and slaughter date. Forestomach weights, MBRT, rumen pH and temperature, estimated polymer accumulation, and log-transformed polymer length were analyzed with the fixed effect of treatment and the random effects of group and slaughter date.

4. Results

4.1. Animal Growth and Health

Average daily grain nor hay intake were affected by the interaction of treatment and sampling day, nor treatment by itself ([Figure 4.1](#)). However, both daily feed intakes increased with time. On an as-fed basis, calves on average during the second acclimation week ate 1.77kg of grain. Average daily grain intake increased by 16% by d0 ($P < 0.01$) and this percent increase was maintained to d7 ($P < 0.0001$). The increase in grain intake slowed to an 11% increase from d7 to d14 at 2.66kg ($P < 0.0001$), after which average daily grain intake was maintained through d28. Meanwhile, average daily hay intake increased throughout the study at a slower rate. Calves ate very little hay during the second acclimation week at only 0.05kg. This increased threefold by d0 ($P < 0.01$), and

then nearly twofold by d21 ($P < 0.01$). By d28, the average daily hay intake reached 0.50kg. Increased feed intake over time was accompanied by increased bodyweight. The bodyweight of bull calves was also not affected by the interaction or treatment by itself. Bodyweight increased linearly from week to week by approximately 5% ($P < 0.0001$; [Figure 4.2](#)). At d-7 calves were 79.01kg, and by d28 increased to 104.55kg with an average daily gain of 0.73kg/d

Regarding calf health, rectal temperature was not affected by any of the fixed effects and averaged 38.1°C. Animals did not demonstrate bloat, scours, absence of fecal matter, reduced feed intake, reduced body weight gain, or abnormal behaviors such as kicking at the stomach and stomping that would have indicated any gastrointestinal blockage due to polymer build-up in the tract. Though, animals did develop signs of bovine respiratory disease shortly after arriving at Virginia Tech. All calves were blanket treated with tulathromycin (Increxxa™, Elanco, Greenfield, IN) and an intranasal bovine rhinotracheitis-parainfluenza 3-respiratory syncytial virus vaccine (Inforce 3®, Zoetis, Parsippany, NJ) by the attending veterinarian. By d0 signs of this disease largely subsided and animals were deemed healthy.

4.2.Hematological Parameters

Hematological parameters were largely unaltered by administration of polymer treatments ([Table 4.1](#)). When comparing blood parameters from d0 to d30 within treatment group, only WBC, neutrophil count, and platelet count seemed to be affected. WBC tended to decrease by 35% in LDPE calves ($P = 0.08$) but did not change for the other two groups. Neutrophil counts tended to decrease by 76% in Control calves ($P = 0.07$) and nearly halved in LDPE calves ($P = 0.03$) but did not change for Blend calves.

Platelet count decreased by 30% for Control calves ($P = 0.01$) and decreased by 40% for LDPE calves ($P < 0.01$) but did not change for Blend calves.

4.3. Rumen Measurements

Rumen measurements are displayed in [Table 4.2](#). Gross rumen measurements of total forestomach weight, reticulorumen weight, empty reticulorumen weight, and total rumen contents did not differ among the treatment groups. Rumen temperature and pH also did not differ among treatments. However, there was a tendency for MBRT to be affected by treatment. Blend calves tended to have reduced MBRT compared to LDPE calves by 46 seconds ($P = 0.06$).

Gross evaluation of epithelium in the cranial dorsal, caudal dorsal, cranial ventral, and caudal ventral regions of the reticulorumen epithelium did not reveal any significant differences between treatment groups ([Table 4.3](#)). In Control and LDPE calves, all regions had no evidence of any damage as indicated by a score of 0. Though one calf in the Blend group demonstrated a very small patch of bare papillae in both dorsal regions of the epithelium. However, these areas were not paired with any scarring, ulcerations, inflammation, or any other indicators of severe damage.

4.4. Papillae Measurements

A treatment and epithelium region interaction existed for the lengths of papillae ($P < 0.01$; [Figure 4.3](#)). Within samples taken from the caudal ventral region of the reticulorumen, papillae of Blend calves were 4.24mm and 50% longer than those of Control calves ($P = 0.04$) but did not differ from those of LDPE calves at 3.56mm. Papillae from LDPE calves also did not differ from those of Control calves which were just 2.84mm long. Papillae length of the cranial dorsal region was not affected by

treatment and averaged 4.33mm. However, papillae from the cranial dorsal region were significantly longer by 61% than those from the caudal ventral region in Control calves ($P < 0.0001$). In gross evaluation of reticulorumen integrity, no apparent disruption of the epithelium was appreciated for any treatment or region. Atrophy, obvious stunting of papillae, necrosis, shredding of stratified epithelium, nor hyperplasia were detected.

4.5. Polymer Residues

In subjective scoring of rumen contents for polymer residues, treatment had a significant influence ($P < 0.01$; [Table 4.4](#)). All Control calves received a score of 0, indicating no polymer presence. Blend calves all received a score of 1 indicating a small amount of degraded polymer present. Half of the LDPE calves demonstrated a score of 3 and the other half demonstrated a score of 4, indicating small and large amounts of intact polymer present within rumen contents, respectively. Polymer residues within rumen contents presented as individual film strips or shards. No apparent aggregation of polymer residues was evident.

This trend was reflected in the estimated polymer accumulation in the rumen and the length of residual polymers. LDPE calves had significantly more polymer residues entrapped in the rumen compared to Blend calves at 27.42g compared to 1.72g ($P < 0.01$; [Figure 4.4A](#)). Calves that received LDPE polymers retained approximately two days' worth of polymer (7% of the total 408g of polymer dosed), while Blend calves retained only 13% of a day's dosage or 0.4% of the total 408g dosed. Lengths of polymer residues were also different between treatment groups ([Figure 4.4B](#)). Polymers retained in the rumens of LDPE calves were 49.51mm long, which was 10 times the length of Blend residues at just 4.81mm long ($P < 0.001$).

5. Discussion

To reduce the incidence of plastic impaction, it is necessary to identify a biodegradable alternative that is not only mechanically suitable to replace polyethylene-based plastics, but safe in the case of animal ingestion. No studies to date have evaluated the potential accumulation and degradation of biodegradable polymer blends if ingested by ruminants. This is a vital step in the process to determine if they are advantageous in protecting ruminant health. Polyhydroxyalkanoates and blends with PBSA may be viable alternatives as they are mechanically similar to polyethylene and are biodegradable by an abundance of biotic and abiotic factors that may be fostered by the rumen environment.

This is the first study to specifically evaluate if a PHA and PBSA biodegradable polymer blend would degrade and pass through the reticulorumen of dairy cattle if ingested compared to a polyethylene control. Twelve Holstein bull calves were administered either a Control treatment of 4 empty gelatin capsules, a Blend treatment of 13.6g of PBSA:PHA melt-blend polymer in 4 gelatin capsules, or a LDPE treatment of 13.6g of LDPE in 4 gelatin capsules for 30d. Animal growth and health were monitored throughout the study and hematological parameters were determined before and after 30d of treatment administration. On d31, animals were sacrificed, gross reticulorumen measurements were taken, epithelium samples were observed for papillae length, and polymer residues were characterized.

Our findings demonstrate that a PBSA:PHA polymer melt blend nor traditional LDPE influence animal health or growth of Holstein bull calves at approximately 0.5% inclusion of the diet, as fed, for 30d. We hypothesized that the LDPE treatment would negatively impact animal health by decreasing the average daily intake of feed and

decreasing weight gain compared to Control and Blend treatments. When plastics and other indigestible foreign materials accumulate within the rumen, they create a sense of fullness in the animal. This is due to increased rumen filling and activation of stretch receptors in rumen epithelium that stimulates satiety centers in the hypothalamus (Baile and Della-Fera, 1981). Decreased feed intake would thereby reduce available energy for the animal and weight gain would theoretically decrease. Though limited studies evaluate plastic impaction of cattle in controlled environments, studies that evaluated animals diagnosed with plastic impaction at abattoirs demonstrate these characteristics. These studies find that animals presenting with plastic foreign bodies are approximately 13% lighter than healthy animals and have a poor appetite (Martín Martel et al., 2021).

Animals similarly demonstrate poor body condition scores highly correlated to increased amounts of plastic materials trapped in the rumen (Tiruneh and Yesuwork, 2010, Berrie et al., 2015, Mahadappa et al., 2020). However, these studies were more observatory and evaluated animals after accumulation occurred over a productive lifetime. The depth at which plastic accumulation influences these parameters and a threshold value before feed intake and body weight decrease in cattle cannot be estimated at this time. Though, a controlled study implanted sheep with 129g, 258g, or 387g of plastic bags via rumenotomy. Results found that sheep implanted with 129g, 0.5% of their body weight, were not significantly affected. However, at 287g, 1.0% of their body weight, feed intake was reduced and body weight decreased by 8.0% after 6wk (Otsyina et al., 2017b). The calves utilized in this study were only 2 months old and presumably not exposed to plastic materials before this study. Therefore, Blend and LDPE calves were only exposed to 408g of polymers that were administered via bolus. Regarding the regulatory pathway

of feed intake through stretch receptors, it is currently unknown how sensitive they are to indigestible materials. Only 27.42g and 1.72g of LDPE and PBSA:PHA were estimated to be maintained in the rumen after 30d of daily ingestion. This is equivalent to 0.3% and 0.02% of total rumen contents (as-fed). Likely, polymer accumulation in this study was not enough to stimulate rumen filling and satiety to reduce feed intake and, subsequently, growth.

Previous observational and controlled studies of plastic impaction in ruminants demonstrate alterations in hematological parameters compared to healthy animals. Observational studies find that WBC (Abd Al-Galil and Akraiem, 2016, Zahra et al., 2017), RBC, and Hb (Akinrinmade and Akinrinde, 2012) are reduced in animals with plastic impaction while MCV and MCHC are elevated (Akinrinmade and Akinrinde, 2012). Another controlled plastic impaction study in which sheep were implanted with plastic via rumenotomy for 42d found at just 129g, or 0.5% body weight, sheep had reduced WBC, RBC, and HB and increased MCV. MCHC was not elevated until 258g of plastic implantation, and platelet counts were increased in animals implanted with 387g (Otsyina et al., 2018). Our study found WBC to change in LDPE calves with a 35% reduction rather than an increase as previously seen. All animals exhibited signs of bovine respiratory disease during the acclimation period. Though animals were treated and deemed healthy to begin the experiment by d0, WBC could have still been elevated from the disease. Initial WBC for LDPE calves was the greatest of the three treatments and slightly elevated compared to a reference value of 8wk old calves (Klinkon and ježek, 2012). However, values still fell into the accepted range of 2.71 to 17.76×10^3 cells/uL as provided by VITALS. It is likely that WBC reduction after 30d was due to

recovery from the disease rather than LDPE administration as the Control and Blend groups also had slight reductions despite not being significant. Neutrophil counts also decreased for Control and LDPE calves after 30d. Although, neutrophil counts remained within the normal range of 0.7 to 6.9×10^3 cells/uL as given by VITALS. As a particular type of WBC, the same trend of decreasing over time is likely explained by the recovery of animals from bovine respiratory disease during the acclimation period rather than a treatment effect. This is especially so as Control animals displayed this trend. Platelet counts similarly decreased after 30d for Control and LDPE calves. Platelets play a key role in the inflammatory response and count increases during times of inflammation throughout the body (Middleton et al., 2016). Bovine respiratory disease is highly associated with inflammation of the respiratory tract. Elevated levels of platelets in this study likely compared to the reference value of 143 to 667×10^3 cells/uL may be due to this inflammatory response. Platelet counts returned to normal values after 30d, indicating recovery of the animals and a lack of rumenitis due to polymer presence. The absence of a true treatment effect, namely LDPE, on changing histological parameters can be explained by the small accumulation of polymer within rumen contents and the short duration of the study. However, it is of note that as a preliminary study, sufficient power was not achieved for any of the histological parameters. Approximately 75 animals per treatment are needed to achieve a power of at least 80% in the analysis of hematological parameters based on the present study.

Gross rumen development was not affected by treatment; total forestomach weight, total reticulorumen weight, and empty reticulorumen weight were not different among treatments. The influence of plastic impaction on rumen growth has not yet been

evaluated in any ruminant species. As previously described, most studies evaluating plastic impaction are post-impaction observations of mature animals when rumen growth and development have ceased. Because polymer accumulation was slight in this study and only for 30d, it is currently unknown if polymer presence would influence rumen development in growing animals. Severe plastic impaction in young animals may lead to reduced feed intake, and therefore decreased available nutrients to support growth of the gastrointestinal tract. However, it is doubtful that severe accumulation of plastics would occur in young animals in standard production conditions in the United States. Total mixed rations contain approximately 0.07% chopped net wrap on an as-fed basis when hay bales are ground with intact netting (Pizol et al., 2017). Thus, accumulation may be slow enough that rumen development would be complete before significant influence by LDPE-based materials. No studies to date have specifically evaluated this. Further studies are needed to specifically evaluate the effect of plastic impaction on rumen growth in developing animals under normal feeding conditions.

Similarly, internal rumen parameters largely did not differ among treatments. Previous observational studies indicate that animals with plastic foreign body impaction typically have increased MBRT and rumen pH compared to healthy animals (Abd Al-Galil and Akraiem, 2016, Al-Galil et al., 2020, Mahadappa et al., 2020). Methylene blue reduction time is an important parameter to evaluate as it is indicative of the fermentation redox potential and activity of microbes in rumen fluid. Healthy animals typically have a MBRT of 180s or less, while animals with plastic impaction may have fourfold increases depending on the level of accumulation (Dirksen and Smith, 1987, Abd Al-Galil and Akraiem, 2016, Al-Galil et al., 2020). This increase in MBRT is due to decreased activity

of microbes, likely due to reduced motility and feed intake of animals with severe plastic impaction (Mahadappa et al., 2020). The average MBRT of Control, Blend, and LDPE calves were all under 180s, and thus it can be concluded that microbial fermentation was not particularly diminished. A potential explanation for reduced MBRT in Blend calves compared to LDPE could be degradation products released from the PBSA:PHA melt blend, more specifically the PHA component. Although we have yet to evaluate specific degradation products released by this polymer in rumen fluid, it is likely that butyrate concentrations within rumen fluid may increase due to the release of 3-hydroxybutyrate from PHA biodegradation (Liu et al., 2019). Depending on the amounts of butyrate released into the rumen pool, total volatile fatty acid concentrations may increase and reduce the rumen redox potential (Huang et al., 2018). More negative rumen redox potential is highly associated with improved microbial fermentation activity (Kalachniuk et al., 1994). It may be through this mechanism that MBRT is reduced in animals dosed with the PBSA:PHA melt blend. However, if volatile fatty acid concentrations were increased in Blend animals, they were not sufficient enough to decrease pH as previously seen in feeding studies of poly(3-hydroxybutyrate) to aquatic species such as European sea bass (De Schryver et al., 2010). As a preliminary study, sufficient power was not met, and a power of test determined that 10 animals per treatment were needed to achieve at least 80% power. Further studies with more animals fed a PBSA:PHA melt blend should evaluate biodegradation products, influence on the microbial ecology, and redox potential of the rumen to sufficiently determine long-term influences on ruminal fermentation.

In gross evaluation of epithelium, observational studies consistently find that animals with plastic foreign bodies present with rumenitis, hemorrhaging, erosion and

sloughing of rumen mucosa, lesions, regions bare of papillae, and bent and stunted papillae (Hailat et al., 1998, Bakhiet, 2008, Otsyina et al., 2017a, Martín Martel et al., 2021). These observations were explained by the aggregation of plastic materials to form ball-like masses that press against rumen epithelium during rumination. These observations were not made in the present study with either Blend or LDPE treatments in any of the four regions of the reticulorumen. Polymer residues in this study did not aggregate together to form masses of material and seemed to be equally distributed among contents. The lack of aggregation of the limited polymer residues likely explains the absence of pathological damage to rumen epithelium and mucosa in the present study. Fully incorporated into rumen contents, polymer residues may not have sufficiently pressed against epithelium to cause irritation or damage. To fully appreciate if a PBSA:PHA blend polymer would induce pathological damages to the rumen compared to a LDPE polymer control, feeding studies of longer duration need to be conducted.

Though gross pathological changes were not observed, papillae length seemed to be influenced by polymer introduction to the rumens of Holstein bull calves in this study. A previous study measured the lengths of papillae from the dorsal rumens of goats and ewes presenting with plastic foreign bodies. Compared to seemingly healthy animals, papillae from affected animals were 60% shorter (Martín Martel et al., 2021). Additionally, papillae length was negatively correlated to the amount of foreign material present within the rumen. In the present study, papillae length within the cranial dorsal region of calves did not differ among those that received either a biodegradable polymer or LDPE compared to animals that did not receive any polymer. The drastic difference in accumulation between the two studies likely explains this. Though the duration of

accumulation in the referenced study is unknown, plastic accumulation was on average 2.75kg (Martín Martel et al., 2021). This is 100 times more than what accumulated in our study over 30d. Interestingly, in the caudal ventral region, Blend calves' papillae were 50% longer than Control calves. As mentioned, it is possible that degradation of the PHA component of the PBSA:PHA melt blend introduces exogenous butyrate into the rumen. Butyrate is a vital modulator in the functional development of rumen epithelium and is involved in cellular proliferation pathways (Sander et al., 1959, Niwinska et al., 2017). As such, butyrate potentially released from this polymer may have stimulated epithelium development and increased papillae length. Though, Blend calves' papillae length in the caudal ventral region were not different from the papillae of LDPE calves. A power test showed that 5 animals are needed to reach at least 80% power. Repeated studies with more animals for longer duration to accrue more polymer residues are necessary to further discern this relationship.

Despite the apparent health of animals in the face of LDPE administration for 30d, characterization of residual polymers in rumen contents indicates PBSA:PHA may be a better material than LDPE for single-use agricultural plastics in the case of ingestion. Animals given 13.6g of LDPE daily had 16 times more polymer residues entrapped in the rumen than those given 13.6g of PBSA:PHA daily. This indicates that accumulation occurs at a much faster rate for LDPE than PBSA:PHA material. However, the accumulation of LDPE was lower than expected. A study in which a total mixed ration containing 0.07% chopped plastic net wrap was fed to cattle for 7 months found that 47.2% of net wrap remains in the reticulorumen in ball-like masses (Pizol et al., 2017). Our study saw only 6.7% of administered LDPE was retained. Given that this polymer is

indigestible, as seen in our previous in situ results (Galyon et al., *in progress*), polymers could have escaped the rumen and been excreted or regurgitated during rumination. The net wrap feeding study also found that plastic particles that were retained in the rumen were more than 70mm long in mature cattle (Pizol et al., 2017) whereas the polymers utilized in this study were only 50mm long. It is likely that some polymers were shorter than the average and escaped through the reticulo-omasal orifice. The lack of aggregation of polymer materials in the rumen also left polymers freely distributed with contents. This could have increased the likelihood of ruminal escape. The absence of aggregation is most likely attributed to the formation of the polymer material in the case of LDPE. Polymer film strips were utilized in this study to better simulate chopped net wrap compared to circular film discs that were used in our previous in situ study (Galyon et al., *in progress*). Though strips could be manually manipulated, they were rather stagnant and did not maintain manipulated shape unless constant pressure was held. Therefore, it is unlikely that film strips could tie together to develop ball-like masses in the rumen as seen throughout plastic impaction cases (Hailat et al., 1998, Bakhiet, 2008, Otsyina et al., 2017a, Pizol et al., 2017, Martín Martel et al., 2021). The absence of aggregation of the PBSA:PHA polymer may better be explained by the breakdown of the material as indicated by reduced fragment size. This reduced residual polymer size likely allowed PBSA:PHA to better escape the ruminal environment and decrease accumulation within the rumen.

It is important to note that this study utilized young Holstein calves, and characteristics of polymer degradation in this study may not entirely reflect degradation in a mature cow. The ruminal microbiome is moldable within the first few months with

the rumen being newly inoculated. It takes approximately 90d for the rumen microbiome to be established (Yin et al., 2021). As the ruminant matures, the microbiome continues to be inoculated with bacteria from the animal's environment, thus providing more opportunities for polymer-degrading bacteria to be hosted. We hypothesize that improved PHA and PBSA degradation will occur in older animals due to increased microbial concentrations and the greater likelihood of PHA and PBSA-degrading bacteria being present. Therefore, it is likely that passage of PBSA:PHA materials through the rumen will be improved in older animals that consume these products.

5. Conclusions

Based on these preliminary findings, feeding of LDPE or PBSA:PHA polymer films at 13.6g/d in Holstein bull calves for 30d does not appear to negatively impact animal health or development. This is largely due to low levels of accumulation at this point and the absence of aggregation that typically induces irritation and damage to the rumen epithelium. Due to the undegradable nature of LDPE in the rumen, LDPE accumulated more than PBSA:PHA. Biodegradable PBSA:PHA degraded to smaller fragments that more readily escaped the reticulorumen. Based on these results, we hypothesize that if cattle consume net wrap developed from PBSA:PHA then the filamentous net wrap material will not aggregate in the rumen and plastic impaction will not occur. However, there are still many parameters to be evaluated before a functional net wrap developed from this biodegradable material can be marketed. Future studies with greater animal numbers should evaluate long-term inclusion of net wrap materials developed from PBSA:PHA in total mixed rations. Histological parameters, rumen development, pathology, and morphology, volatile fatty acid concentrations, polymer

degradation products, rumen fluid redox potential, distribution of net wrap throughout the entire gastrointestinal tract, and net wrap passage rate should all specifically be determined.

6. Tables

Table 4.1. Hematological parameters of Holstein bull calves at 0d (Initial) and 30d (Final) of administration of either 4 empty gelatin capsules (Control), 13.6g of a Poly(butylene succinate-co-adipate) and Polyhydroxyalkanoate melt-blend in 4 gelatin capsules (Blend), or 13.6g of Low-density Polyethylene in 4 gelatin capsules (LDPE).

Parameter	Control		Blend		LDPE		SEM	P-value		
	Initial	Final	Initial	Final	Initial	Final		¹ Trt	² Ti	Trt X Ti
RBC, cells/uL	10.63	10.33	11.57	11.24	10.54	10.71	0.50	0.25	0.66	0.80
HB, g/dL	10.68	10.80	11.30	11.35	10.95	11.50	0.48	0.54	0.46	0.79
Hematocrit, %	36.03	35.80	38.03	37.23	36.65	38.18	1.48	0.47	0.89	0.72
MCV, fL	33.88	34.78	32.90	33.10	34.83	35.65	0.68	0.02	0.06	0.62
MCHC, g/dL	29.60	30.18	29.70	30.50	29.85	30.13	0.36	0.83	0.06	0.74
RDW, %	27.63	26.18	27.95	27.75	26.88	26.33	1.16	0.72	0.03	0.26
Reticulocyte, cells/uL	0.85	1.75	1.15	1.98	1.80	1.63	0.58	0.81	0.16	0.40
WBC, cells/uL	9.67	8.13	9.19	8.43	10.17	7.55*	0.96	1.00	0.01	0.35
Neutrophil, cells/uL	4.48	2.55*	4.48	3.00	4.50	2.23 ^a	0.70	0.82	<0.01	0.68
Lymphocyte, cells/uL	3.73	4.05	3.53	3.90	3.95	4.03	0.39	0.83	0.17	0.77
Monocyte, cells/uL	1.30	1.40	1.03	1.40	1.53	1.18	0.17	0.59	0.72	0.06
Eosinophil, cells/uL	0.00	0.03	0.05	0.03	0.08	0.00	0.03	0.69	0.23	0.15
Basophil, cells/uL	0.10	0.15	0.10	0.13	0.13	0.10	0.02	0.74	0.29	0.16
Platelets, cells/uL	684.75	526.00 ^a	703.50	643.50	681.75	486.50 ^a	33.56	0.10	<0.01	0.08
MPV, fL	7.03	7.15	7.30	7.38	7.18	7.05	0.22	0.42	0.81	0.57
ESR, mm/24h	4.52	4.34	4.98	4.08	4.11	4.11	0.49	0.50	0.35	0.59

¹Treatment effect

²Sampling time effect

^a Indicates a significant difference from the initial value within treatment, $P < 0.05$

* Indicates a tendency to differ between time points within treatment, $P < 0.10$

Data shown are the least squared means.

Table 4.2. Rumen parameters of Holstein bull calves after 30d of administration of either Control, Blend, or LDPE treatment.

Parameter	Control	Blend	LDPE	SEM	P-value
Forestomach, kg	16.04	14.57	16.19	1.29	0.24
Reticulorumen, kg	12.44	11.58	13.02	1.20	0.25
Empty reticulorumen, kg	2.11	2.08	2.20	0.18	0.63
Rumen contents, kg	10.33	9.50	10.82	1.09	0.35
MBRT, s	143 ^{ab}	108 ^b	154 ^a	17	0.06
Rumen pH	6.8	6.8	6.9	0.1	0.61
Rumen temperature, °C	37.8	38.3	38.3	0.7	0.58

^{a,b} Different letter superscripts indicate tendencies for treatments to differ ($P < 0.10$)

Data shown are the least squared means.

Table 4.3. Frequency (%) of gross pathology scores of rumen epithelium in the cranial dorsal (CrD), caudal dorsal (CaD), cranial ventral (CrV), and caudal ventral (CaV) regions of Holstein bull calves after 30d of administration of either Control, Blend, or LDPE treatment.

¹ Score	Control				Blend				LDPE			
	CrD	CaD	CrV	CaV	CrD	CaD	CrV	CaV	CrD	CaD	CrV	CaV
0	100	100	100	100	75	75	100	100	100	100	100	100
1	0	0	0	0	25	25	0	0	0	0	0	0
2	0	0	0	0	0	0	0	0	0	0	0	0
3	0	0	0	0	0	0	0	0	0	0	0	0
4	0	0	0	0	0	0	0	0	0	0	0	0
5	0	0	0	0	0	0	0	0	0	0	0	0

¹Gross pathology was scored using a 6-point scoring system: 0 = no evidence of any damage, 1 = palm-size or smaller areas bare of papillae, 2 = large areas bare of papillae, 3 = areas of scarring, 4 = red/bloody areas, 5 = areas of parakeratosis.

For both CrD and CaD regions, Treatment: $P = 0.34$.

Table 4.4. Frequency (%) of rumen content scores of Holstein bull calves after 30d of administration of either Control, Blend, or LDPE treatment.

¹ Score	Control	Blend	LDPE
0	100	0	0
1	0	100	0
2	0	0	0
3	0	0	50
4	0	0	50

¹Rumen content was scored using a 5-point scale: 0 = no polymer present, 1 = small amount of degraded polymer present, 2 = degraded polymer present throughout contents, 3 = small amount of intact polymer present, and 4 = intact polymer present throughout contents.

Treatment: $P < 0.01$

7. Figures

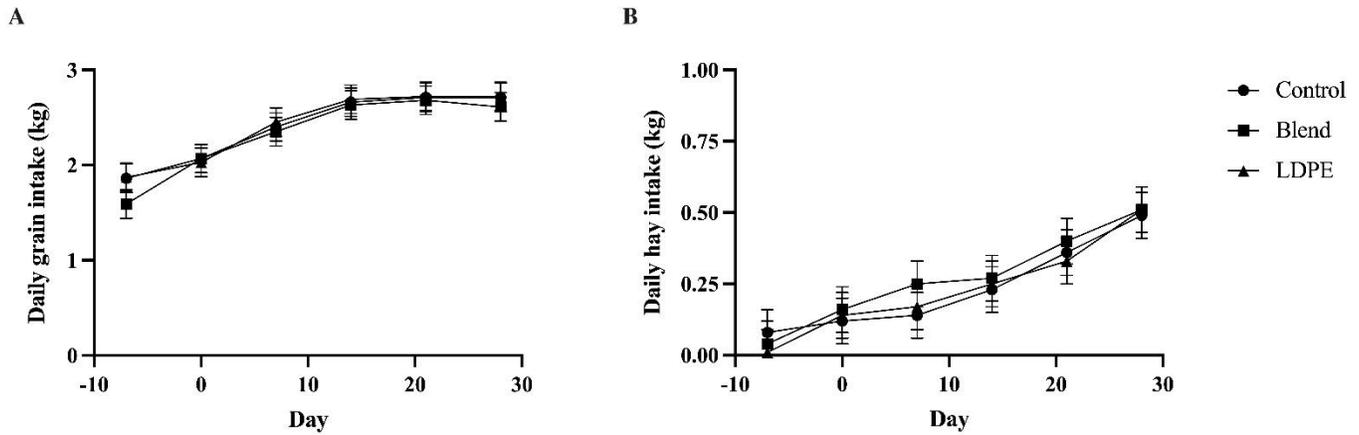


Figure 4.1. Average daily feed intake determined on a weekly basis, as fed, of Holstein bull calves given either empty gelatin capsules (Control), 13.6g of a Poly(butylene succinate-*co*-adipate) and Polyhydroxyalkanoate melt-blend (Blend), or 13.6g of Low-density Polyethylene for 30d, separated by average daily grain intake (A) and average daily hay intake (B). For grain intake, Treatment: $P = 0.28$; Day: $P < 0.01$; Treatment x Day: $P = 0.60$. For hay intake, Treatment: $P = 0.73$; Day: $P < 0.01$; Treatment x Day: $P = 0.82$. Data are shown as least squared means of treatments within time (LSM \pm SEM).

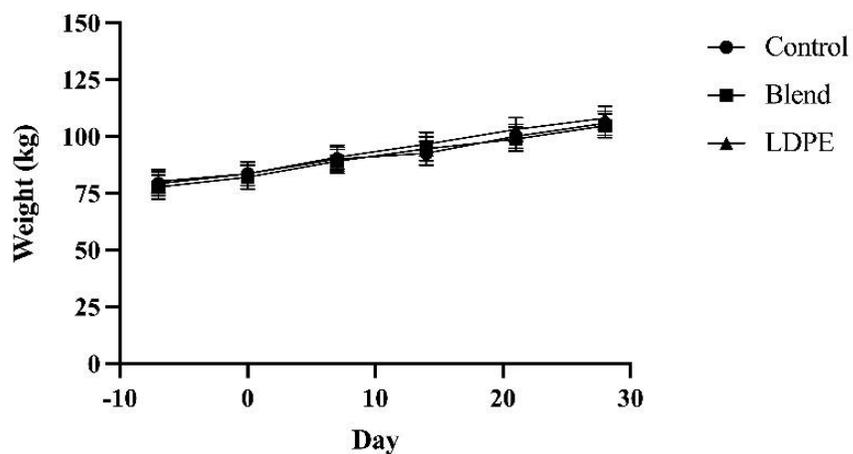


Figure 4.2. Weekly body weights of Holstein bull calves given Control, Blend, or LDPE treatments for 30d. Treatment: $P = 0.69$. Day: $P < 0.01$. Treatment x Day: $P = 0.15$. Data are shown as least squared means of treatments within time (LSM \pm SEM).

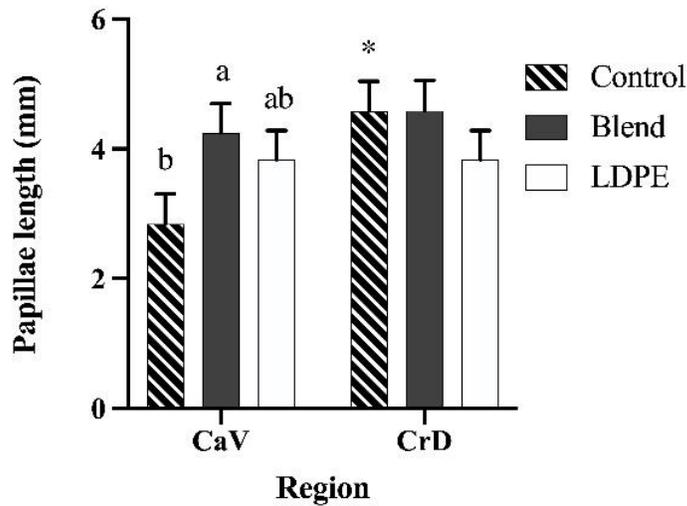


Figure 4.3. Papillae length in the caudal ventral (CaV) and cranial dorsal (CrD) regions of the reticulorumens of Holstein bull calves after 30d of Control, Blend, or LDPE treatment administration. Treatment: $P = 0.19$. Region: $P < 0.0001$. Treatment x Region: $P < 0.001$. ^{a,b} Different letters indicate differences between treatments within epithelium region. * Indicates a significant difference between regions within treatments. Data are shown as least squared means of treatments within region (LSM \pm SEM).

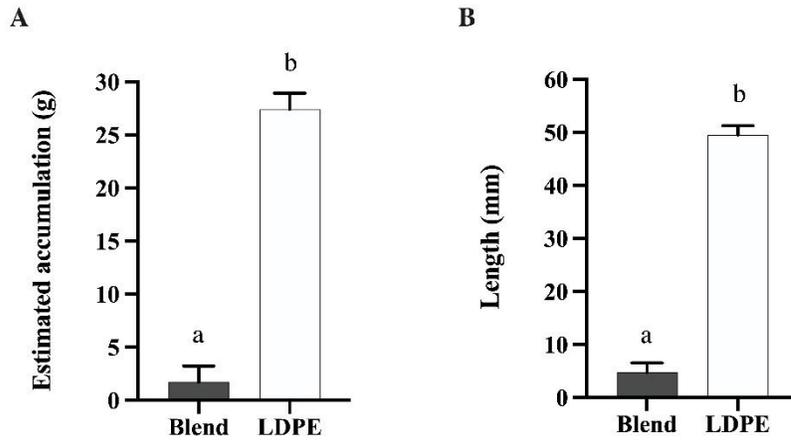


Figure 4.4. Characterization of polymer residues within rumen contents of Holstein bull calves given either Blend or LDPE treatment for 30d, separated by: **A)** estimated total polymer accumulation, and **B)** average polymer length. For both parameters, Treatment: $P < 0.0001$. ^{a,b} Different letters indicate differences between treatments. Data are shown as least squared means of treatments (LSM \pm SEM).

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CHAPTER 5

CONCLUSION AND IMPLICATIONS

1. Summary and Future Studies

Although mass loss and the degradation rate of polymers that underwent *in vitro* fermentation in rumen fluid were quite low, thermochemical analyses indicated that the beginning stages of degradation might have occurred within 24h. Increasing melting temperature and decreasing onset thermal degradation temperature could have indicated the shortening of polymer chains induced by preferential depolymerase degradation of amorphous material. True degradation was seen when PHA, PBSA, PBSA:PHA, and LDPE were incubated in the rumens of three cannulated, Holstein dairy cattle. A melt blend of PBSA and PHA fragmented within just 1d, whereas PHA took 14d, PBSA took 60d, and LDPE never degraded. This early fragmentation of the polymer may allow the material to pass through the reticulo-omasal orifice and through the rest of the gastrointestinal tract. This was demonstrated by administering daily boluses to calves for 30d. When either PBSA:PHA or LDPE films were administered to animals, they did not demonstrate any symptoms of gastrointestinal blockage throughout the study likely due to the short-term nature of the experiment. However, LDPE films did not appear to degrade at all and accumulated within the reticulorumen to a greater extent than PBSA:PHA, which degraded into fragments 10% of their original size. From these preliminary studies, we propose utilizing PBSA:PHA for single-use materials for feed storage may be a better alternative to LDPE due to its degradation and passage from the reticulorumen. This may reduce long-term plastic accumulation in domestic cattle.

However, there are still many parameters to be evaluated before a functional net wrap developed from this biodegradable material can be marketed. Future studies with greater animal numbers should evaluate long-term inclusion of net wrap materials developed from PBSA:PHA in total mixed rations. In comparison to LDPE materials, animal feed intake, growth, and health should be evaluated. Additionally, histological parameters, rumen development, pathology, and morphology, volatile fatty acid concentrations, polymer degradation products, rumen fluid redox potential, distribution of net wrap throughout the entire gastrointestinal tract, and net wrap passage rate should all specifically be determined. Should this material truly pose no health complications for animals by its degradation, and the material maintains very low accumulation levels, PBSA:PHA is a viable alternative to LDPE materials, pending its mechanical applicability.