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**Functional dietary diversity as opposed to monotony: effect on  
animal production, welfare, and environmental impact**

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A thesis

submitted in partial fulfilment  
of the requirements for the Degree of  
Doctor of Philosophy

at

Lincoln University

by

Konagh Garrett

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# Manuscripts prepared, submitted, and accepted arising from this thesis

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**Chapter 4:** Garrett, K., M. R. Beck, C. J. Marshall, A. E. Fleming, C. M. Logan, T. M. R. Maxwell, A. W. Greer, and P. Gregorini. 2021. Functional diversity vs. monotony: the effect of a multiforage diet as opposed to a single forage diet on animal intake, performance, welfare, and urinary nitrogen excretion. *J Anim Sci.* 99(5). doi: 10.1093/jas/skab058.

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**Abstract of a thesis submitted in partial fulfilment of the requirements for the Degree of Doctor of Philosophy.**

Functional dietary diversity as opposed to monotony: effect on animal production, welfare, and environmental impact

by

Konagh Garrett

The hypothesis of my research was that providing functional diversity as opposed to dietary monotony will: alter fermentation patterns, increase animal production, and reduce negative environmental impacts, enhance animal welfare, and alter neophobia and partial preference through *in utero* and early life exposure. Thereby the objective was to determine the effects of altering the functionality of diverse diets (through context, species abundance, species distribution, and temporal availability) on dry matter intake, production, welfare, the environmental impacts, and partial preference compared with animals grazing a monotonous diet. This research was conducted over several experiments. Chapter 3 implemented an *in vitro* rumen fermentation methodology to determine if diverse versus monotonous diets altered fermentation patterns and allowed for assumptions on production and environmental impacts. The results showed that increasing the portion of chicory, plantain, or a diverse combination (chicory, plantain, and alfalfa) to ryegrass increased 24 hr gas production and branched-chain volatile fatty acid production, while reducing ruminal ammonia concentration. Chapter 4 applied an equal parts dry matter (DM) diverse combination diet to ram lambs and compared their intake, performance, welfare, and urinary nitrogen excretion to those offered a repetitive ryegrass diet. Lambs grazing the diverse diet had a 48% greater dry matter intake (DMI), 92% greater average daily gain (ADG), 25% lower day-to-day coefficient of variation (CV) of intake, and had a 30% lower urinary N concentration. Ram lambs provided a varied diet with set ratios of each species had a 20 and 10% greater DMI and a 29 and 23% reduced DMI CV compared to a diverse diet of the same ratios and a ryegrass diet that were monotonous in presentation in the experiment in chapter 5. The experiment in Chapter 6 explored the diverse and varied diet treatments without the restrictions of set ratios of species and compared them to a monotony of alfalfa. At the same level of intake the varied diet lambs gained 67 and 28% greater than the diverse and alfalfa diet. This greater ADG of the varied lambs occurred with the same intake and diet primary chemistry as the diverse diet, indicating that performance was affected by more than primary chemistry. Lambs consuming the alfalfa treatment conducted 150% more

bouts of stereotypic behavior than the diverse and varied lambs. Chapter 7 provided ewes in the final third of gestation with diverse or monotonous ryegrass diets. I found that diverse ewes birthed heavier lambs and exhibited lower levels of oxidative and metabolic stress 24 hrs after lambing. In chapter 8 the lambs birthed in chapter 7 remained with their dams on their respective treatment until weaning (early life experience) or were removed 24 hrs after lambing to pinpoint the effect of *in utero* and early life on partial preference and neophobic behaviors in later life. Lambs with early life exposure had partial preference altered more than those with only *in utero* exposure. Lambs exposed to ryegrass *in utero* or *in utero* and in early life spent more time grazing ryegrass than their diverse counterparts. In general, the diverse lambs had reduced latency to graze the diverse species compared to the ryegrass lambs. Further, all lambs chose to comprise a mixed species diet. This chapter demonstrated how *in utero* and early life experience can reduce neophobic behavior and that when provided the choice animals prefer to comprise a diverse diet even if a familiar forage species is available.

**Keywords:** Diverse, monotony, diet, animal production, animal welfare, environmental impact

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Finally, a short thesis for those that told me I never would and those that never could.

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# Chapter 1

## Introduction

The nutrition supplied to farmed ruminants impacts most parts of production systems, including animal growth, health, welfare, production, product quality, the environmental impact and financial viability of farming practices (Vasta et al., 2008; Vasta and Luciano, 2011; Gerber et al., 2013; Vazirigohar et al., 2014; Makkar, 2016). All these aspects of livestock production systems are under increasing societal and economic pressures as increased production is demanded from systems alongside reduced negative environmental impacts and improved animal welfare (Foot et al., 2015; Gerber et al., 2013; Gregorini et al., 2017). With nutrition's wide ranging ramifications it is surprising that conventional pastoral feeding systems still feed simple one-size-fits-all diets (e.g. ryegrass and white clover), fed to meet the 'average' animals nutrient requirements. This occurs despite the known individualistic nutritional requirements of animals. Individual's nutritional requirements are based on a culmination of their unique genetics, physiology, morphology, and preferences as determined by past experiences, current nutritional and energetic state (Provenza et al., 1996; Scott and Provenza, 1999; Manteca et al., 2008; Beck and Gregorini, 2020). With differences in and continuously changing nutritional requirements, it is not surprising that a single feed would fail to act as a balanced sole diet component for a range of individuals through time. Yet, such diets are often offered in monotonous arrangements for ease of pasture or feed management, despite that they may impair productivity, increase environmental footprint, and diminish animal welfare through nutritional and sensorial deprivation (Champion et al., 1998; Scott and Provenza, 1999; Rogosic et al., 2006; Distel et al., 2007; Provenza et al., 2007; Dixon and Pasinetti, 2010; Villalba et al., 2011; Beck and Gregorini, 2020)

Alternatives to simple repetitive one-size-fits all monocultures or homogenously mixed pastures are encompassed within diverse diets. Diversity itself encompasses a) species or character **richness**, b) the **abundance** of each species or character within the area of interest, c) the **distribution** of the species or character throughout the specified area, d) the **individuality** or **variety** of the species or characteristic as a result of genetic variation, epigenetics, resource availability, and the ability of each individual or their organs to utilize that resource, e) how a-d vary through the **temporal** scale (Tilman et al., 1997; Purvis and Hector, 2000; Mason et al., 2005; Mouillot et al., 2005), and f) any interactions between (a-e). If the components a-f individually or in combination remain static through a set temporal scale, diverse diets can contain a monotonous component or be provided in a monotonous manner. Thereby diversity can be provided in a more functional manner to minimize or mitigate monotony, where the presentation or arrangement of dietary components in

biologically significant quantities such that the animal can choose and fulfill its individual nutritional and eudemonic requirements through time and space. I dub this functional diversity. Providing farmed livestock with dietary diversity (richness in flavor or chemical composition) increases dry matter intake or feed conversion efficiency (Champion et al., 1998; Rogosic et al., 2006; Distel et al., 2007; Villalba et al., 2011), animal health (Provenza et al., 2007; Dixon and Pasinetti, 2010), and productivity (Rodríguez et al., 2007; Al-Marashdeh et al., 2020). Although, these promising effects of dietary diversity compared to simple monotonous diets have been identified, much of this research has been conducted using concentrates or conserved forage and diversity with low-functionality with little information regarding the effect of fresh forages.

The proposed Ph.D. research uses sheep as a ruminant model to determine the effects of altering the functionality of diverse diets (context, species abundance, species distribution, and the temporal availability) on animal intake, production, welfare, and the environmental impacts compared with those grazing a monotonous diet. Following this introduction, this thesis is composed of a literature review defining functional dietary diversity and the tools that can be used to design or incorporate it into systems, which has been submitted to an international peer-reviewed journal (Chapter 2). There are six experiments described in manuscripts prepared for submission, submitted in review, and accepted in international peer reviewed journals (Chapters 3-8), and finally a general discussion (Chapter 9). The three central hypotheses for this thesis are based on providing functional diversity as opposed to monotonous diet repetition will: 1) alter fermentation patterns, increase animal production, and reduce negative environmental impacts, 2) enhance animal welfare, 3) alter the foraging behavior and partial preference through *in utero* and early life exposure to the given diets. Hypothesis one was broken into three subsidiary hypotheses, where providing functional dietary diversity as opposed to a monotonous single forage diet will: a) alter fermentation patterns b) increase dry matter intake and animal production c) reduce the negative environmental impact of animal practices. Hypothesis 1a is explored in Chapter 3 using *in vitro* rumen fermentation rates and characteristics and later in chapters 5 and 6. Hypothesis 1b is explored in all experimental chapters (3-7) with a range of diverse diets with differing functionalities and a range of monotonous diets. Hypothesis 1c is explored in chapters 3, 4, 5, and 6 by comparing the ruminal characteristics, metabolism, growth and feed conversion efficiency data collected from functionally diverse and monotonous diets. Hypothesis 2 is explored in chapters 4, 5, 6, and 7 exploring a range of parameters indicative of welfare (intake stability, production, behavior, and blood constituents). The last hypothesis (3) is tested in chapter 8 where preference of lambs exposed to functionally diverse or monotonous diets *in utero* or in early life (*in utero* to weaning) were compared approximately 3.5 months after weaning. Further, as a range of functionally diverse diets and diverse diets provided

monotonously were compared, my thesis tested hypotheses related to the importance of diverse diets provided in a functional manner.

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## Chapter 2

### Literature Review: Heraclitus's dilemma engrained in grazing systems: conceptualization of functional diversity

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\*External Contribution

**Author Contributions:** Conceptualization, K.G., J.V, N.T., and P.G.; methodology, NA.; software, K.G.; validation, NA; formal analysis, K.G.; investigation, K.G.; resources, K.G.; data curation, K.G.; writing—original draft preparation, K.G.; writing—review and editing, K.G., J.V, N.T., T.M.R.M and P.G., and P.G.; visualization, K.G.; supervision, K.G., J.G, N.T., and P.G.; project administration, K.G.; funding acquisition, K.G.

## 2.1 Abstract

Livestock production systems are facing increasing societal pressure to improve animal welfare and reduce the environmental impact of practices while simultaneously meeting production demands in an economical manner. Currently there is a wide uptake of a one-size-fits-all management approach in feeding for the 'average' animal within and across pastoral livestock systems, which is fueling the environmental and welfare concerns of society and consumers. One-size fits all approaches are used despite knowledge of the varying individuality of animal nutritional requirements, determined by their morphology and physiology, genetics, epigenetics, sex, developmental stage, nutritional state, and experience within the specified physical and social settings. As nutritional requirements are wide-ranging between and within individuals through time, it is not surprising that no one diet or plant could act as the sole constituent of a "balanced-diet" for ruminants which are known to utilize a diverse array of plants when afforded the opportunity. Even with this knowledge pastoral based systems still feed simple-mixes or single forage diets aiming to meet the average nutritional requirements of the herd and allocate these feeds repetitively, providing livestock with a monotonous diet. Monotony was stated to be an unnatural condition for living things two and a half thousand years ago by the Greek philosopher Heraclitus of Ephesus. As such monotonous single or simple mix feed/forage diets now engrained within systems have since been shown to cause a plethora of issues by instilling boredom, nutrition related health, welfare, environmental, and productivity issues. Dietary diversity is an alternative being explored to provide a more balanced and individualized diet to the animal. However, there is an overlap between dietary diversity and monotony, in that a diverse diet can be monotonous, thereby there is a need to provide a term and concept that describes the provision of a diverse diet in the absence of monotony, which we term functional diversity. The primary objective of this review is to outline functional diversity and how the spatiotemporal arrangement of feeds and forages can provide benefits to production, health and welfare, and the environment. Further, there is the challenge for farm management or designers of future productive foodscapes to know what tools can be used to alter the functionality of the diverse diet on offer. Thus, we present a model that illustrates the variables that can alter the functionality of the diverse diet and the tools that we can use to manipulate these variables.

**Key words:** Grazing, Functional Diversity, Monotony, Diversity



## 2.2 Introduction

Livestock production systems navigate a difficult course between meeting production demands economically while simultaneously addressing societal pressures to reduce their environmental impacts (Gerber et al., 2013; Foote et al., 2015; Gregorini et al., 2017) and improve animal welfare (Gregorini et al., 2017; Leroy, 2019). Intensification, furthermore, has led to realization that a one-size-fits-all management approaches within and across our pastoral livestock systems generates practices such that are inherently inefficient. This is especially apparent regarding animal nutrition.

Animal nutrition impacts almost every aspect of animal production systems, including animal health, growth, welfare, production, product quality, environmental footprint, and financial viability (Vasta et al., 2008; Vasta and Luciano, 2011; Gerber et al., 2013; Makkar and Ankers, 2014; Vazirigohar et al., 2014; Makkar, 2016). Individual nutrient requirements and feed preferences of livestock are determined by their morphology and physiology, personality, genetic variation, phenotypic variation, epigenetics, sex, developmental stage, nutritional state, experience (individual and social learning) and physical and social settings (Provenza et al., 1996; Scott and Provenza, 1999; Dall et al., 2004; Manteca et al., 2008). However, despite knowledge of these important influences we often feed for the average animal and if requirements for particular nutrients are normally distributed across a population, it follows that diets that conform to the requirements of the 'average' animal will inevitably fail to meet the requirements of animals at either tail of the distribution around the mean (Beck and Gregorini, 2020). For example, the within-herd or between breed daily metabolizable energy requirement for maintenance can vary as much as 35%; thereby, cows 1 standard deviation above the mean body weight of 500 kg (approximately 16.5% of the population) would require at least an additional 2.9 Mcal per day (DiCostanzo et al., 1990), highlighting that over and underfeeding is common in usual herd feeding practices. It is a paradox therefore that while research has revealed persistent individual differences in nutritional requirements and feeding preferences, diets offered within livestock production systems are generally modeled off the nutrient requirements of the 'average' rather than the individual animal (Scott and Provenza, 1999; Atwood et al., 2001; Beck and Gregorini, 2020).

Ancestors of today's average fed farmed ruminants and today's wild and domestic livestock grazed or graze selectively from a broad range of biochemically diverse plant species to meet their individual nutrient, medicinal and prophylactic requirements (Provenza et al., 2003). Further, today's ruminant species display a range of feeding types, levels of selectivity, and grazing behaviors.

However, when afforded the opportunity they all generally choose a mixed diet (Hofmann, 1989). Within managed grazing settings animals are often not given the opportunity to select from multiple diet components. Thereby, in addition to being fed a diet suitable in composition and quantity for the herd 'average', animals within intensive systems are fed diets of low diversity, constraining natural preference behaviors. Such one-size-fits-all simple diets (e.g. grass-clover pastures) provide advantages, in virtue of easy grazing or feed management (Flores, 2020), as plant-specific fertilization, weed management regimes (Pembleton et al., 2015; Distel et al., 2020; Flores, 2020), and defoliation interval and intensity only require the consideration of a small number of species (Pembleton et al., 2015). As a result monocultures or simple legume-grass associations are often incorporated across the entire farm platform, thereby providing animals with a monotonous diet. Monotony is defined as 'a lack of variety or interest; tedious repetition or routine; the quality of being dull, unvarying' (OED, 2021). Dietary monotony means repetition of effectively invariant biochemical or sensorial input in both space and time.

The state or dilemma of monotony was conceived by the Greek philosopher Heraclitus of Ephesus (c. 535 – c. 475 BC). He argued that monotony is an unnatural condition for living things (which holds true for herbivores), which have the intrinsic capacity to mediate their homeostasis within a continuously changing environment when afforded the tools they require to do so (i.e. dietary choice). Two and a half thousand years later, ruminants are fed in intensive pastoral production systems in a manner that matches the traditional definition of specialists that consume one or a small number of food items in their natural habitats (Shiple et al., 2009). It follows that enforcing the unnatural condition of monotony onto farmed ruminants through diet would have disadvantages, such as boredom (Burn, 2017; Villalba and Manteca, 2019), that reduces intake, growth, production, and welfare at the eudemonic (happiness through the pursuit of purpose) and hedonic (happiness through pleasure) levels (Beck and Gregorini, 2020). The unnatural condition of monotony can violate four of the five freedoms often used to assess animal welfare: Freedom from (1) thirst, hunger, and malnutrition, (2) discomfort, (3) pain, injury, and disease, (4) to express normal behavior (Farm Animal Welfare Council, 1993; Webster, 2016). Examples of detrimental phenomenon that can be induced by monotonous diets are incidental restriction and augmentation, which are respectively the restriction of intake due to negative post-ingestive feedback of consuming a specific nutrient and the over ingestion of nutrients to fulfill requirements for other nutrients or energy [see Gregorini et al. (2017) and Villalba et al. (2015b)]. Furthermore, monotonous presentation of a nutritionally balanced diet has been linked to sensory-specific satiety where the repeated presentation of a food generates oro-sensorial experiences (e.g. textural or flavoural) that trigger satiety (Rolls et al., 1982; Blundell et al., 1994; Provenza et al., 1996; Villalba et

al., 2011a; Blundell and Bellisle, 2013; Gregorini et al., 2017). For example, Villalba et al. (2011) found the intake of lambs offered a selection of four flavored rations, each identical in nutritional composition, was greater than that of lambs offered just one of four ration flavors. As a result of our growing scientific appreciation of the importance of diverse and varied diets there is an emerging interest in alternatives to dietary monotony.

An alternative to dietary monotony is a diet offering a diversity of diet components, i.e. a diverse diet. Diversity is the “condition or quality of being diverse, different, or varied; difference unlikeness” (OED, 2021). In terms of biological communities and animal nutrition it encompasses (Tilman et al., 1997; Purvis and Hector, 2000; Mason et al., 2005; Mouillot et al., 2005): (a) species or character **richness** (i.e number of species at a site), (b) the **abundance** of each species or character at a given site (space, spatial scale), (c) how species or a character are **distributed** throughout space, i.e. that site; and d) within species or characteristic **individuality** or **variety** as determined by intraspecific genotypic or phenotypic variation in the ability of each individual/organ to utilize the given resources, (e) how qualities (a-d) vary through time (**temporal scale**) and (f) interactions between (a-d). The latter includes, for instance, the way in which distribution of feeds in space influences the ability of an animal to achieve temporal variation in the composition of its diet.

Descriptions of diversity must specify (a-f) within the context or a situational description of what diversity is considered. Without such a description, the term diversity is either meaningless or can be openly interpreted by readers as any component or combination of (a-f). Due to this intrinsic complexity of diversity, and the orthogonal (i.e. varying independently of one another) nature of its components, it is not possible to attribute a single numerical value to completely represent the diversity of a community (Legovic, 1991; Mouillot et al., 2005; You et al., 2009), in our context, the diversity of feeds. Several arithmetical approaches are used to describe individual components of richness, abundance, and evenness at a given site or a combination of two components (e.g. Shannon Wiener’s diversity index is a combined measure of species richness and their relative abundance) (Shannon, 1949; Simpson, 1949; McIntosh, 1967; Berger and Parker, 1970; Mason et al., 2003; Villéger et al., 2008). Arithmetical approaches such as these provide detailed inference about community composition that includes more than a single component of diversity (Kim et al., 2017), but fails to capture all facets (a-f) of diversity within a single value. Variety is a term often used synonymously with diversity, although variety is a component of diversity that describes the within species or characteristic individuality [see (d) for full description]. Therefore, it should not be used synonymously in dietary descriptions to avoid confusion.

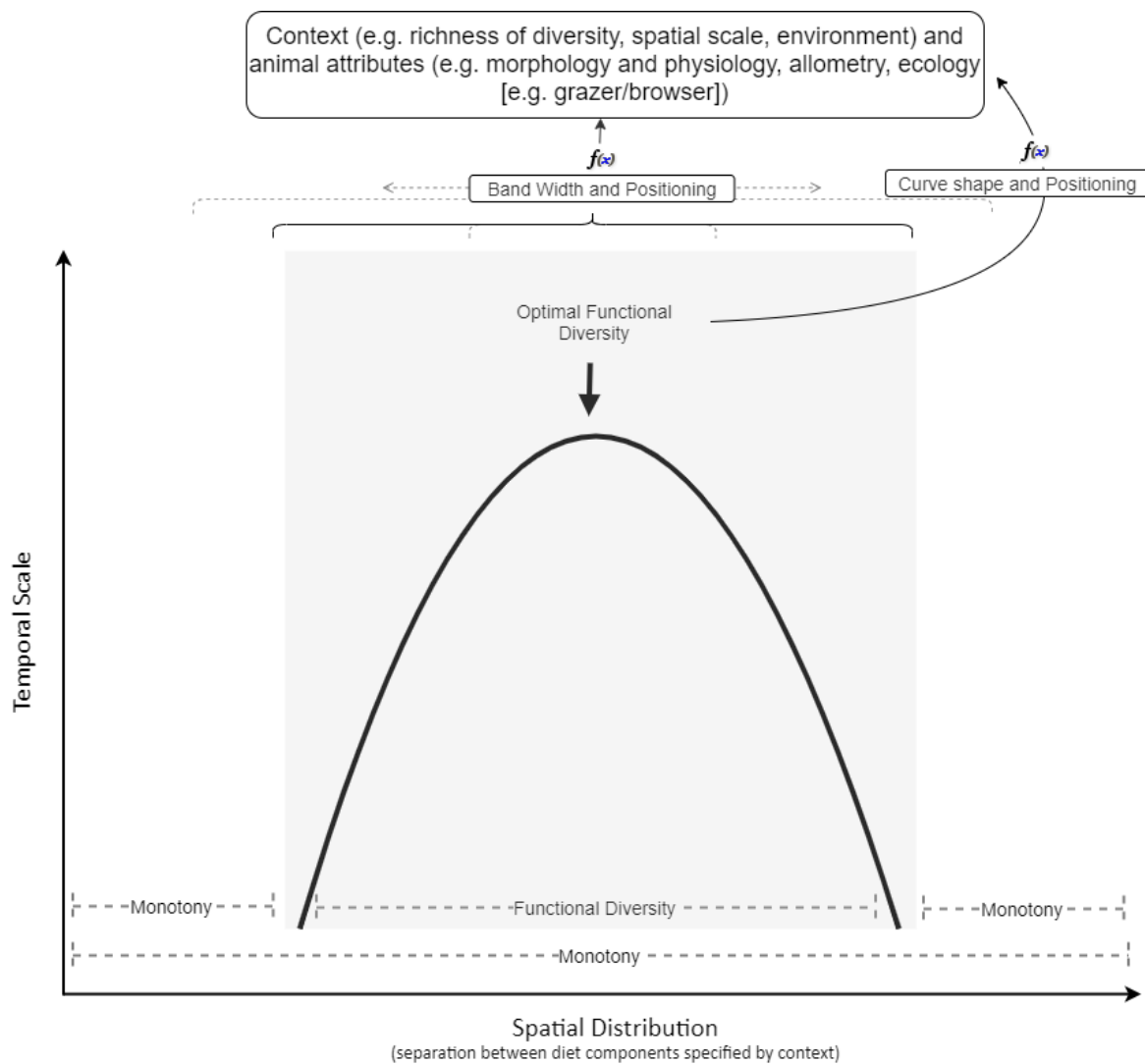
Diversity, however, is not 'immune' to monotony, as if any component or combination of (a-f) remains constant, or near-constant, within a set time period, such a diverse diet can contain a level of monotony. The ability of diversity to contain a monotonous facet highlights the overlap between the terms and the need to describe and develop a continuum or concept that recognizes the overlap between diverse and monotonous diets and identifies the multitude of ways diversity can be manipulated to provide a 'best-fit' alternative within a context. From here emerges a need for a term that encompasses diversity without the constraints of monotony and therefore we propose the concept 'functional diversity'. The requirement for such a term and dietary design to incorporate such a concept is evidenced by the decisions made by foraging or grazing animals offered choice to meet individualistic nutrient requirements, where the currency alongside an animal's current state, the environment state, and behavioral strategies that optimize fitness (long-term contribution to the gene pool) influence the decisions made (the unified foraging theory) (Mangel and Clark, 1999).

Hereon in, we present the concept and a conceptual model of functional diversity. Second, we present empirical evidence to support and illustrate our argument for the definition of functional diversity rather than diversity. In addition, we describe benefits to production, health and welfare, and the environment as a result of incorporating functional diversity into grazing systems. Finally, we illustrate the variables and tools that management can use to alter the functionality of a diverse diet to provide the benefits outlined.

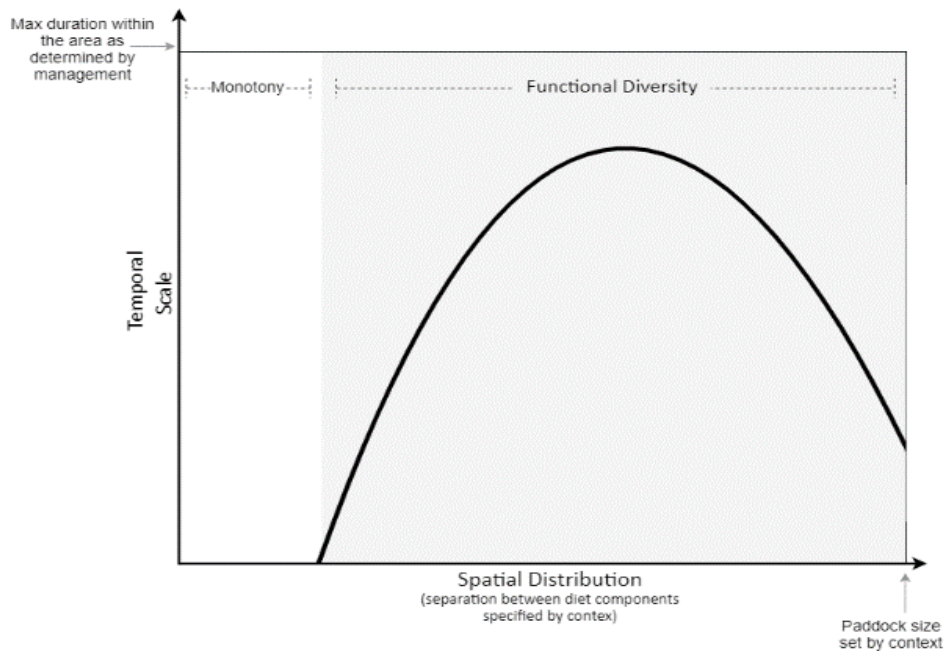
### **2.3 Functional diversity**

An animal's ability to comprise a diverse diet without the constraints of monotony is a function of the scales (temporal and spatial distribution of feeds) and context (external and internal context e.g. environmental conditions, paddock size, animal species, and the internal state of the animal) of grazing. Thereby, **functional diversity** is the presentation or arrangement of dietary components in biologically significant quantities such that the animal can choose and fulfill its individual nutritional and eudemonic requirements through time and space (Figure 2.1). 'Biologically significant' can be considered in two dimensions. The first is context specific (both external and internal context e.g. setting and animal type) and refers to the quantity of a single nutrient within that feed required to elicit an effect (i.e. contributing to meet a nutrient requirement) or the quantity that provides a divergent biochemical and oro-sensorial input stimulating motivation to eat (Figure 2.1). Further, although set by the context of the situation, the richness and feed components paired or presented within one diet can provide greater functionality than another diet, in that the

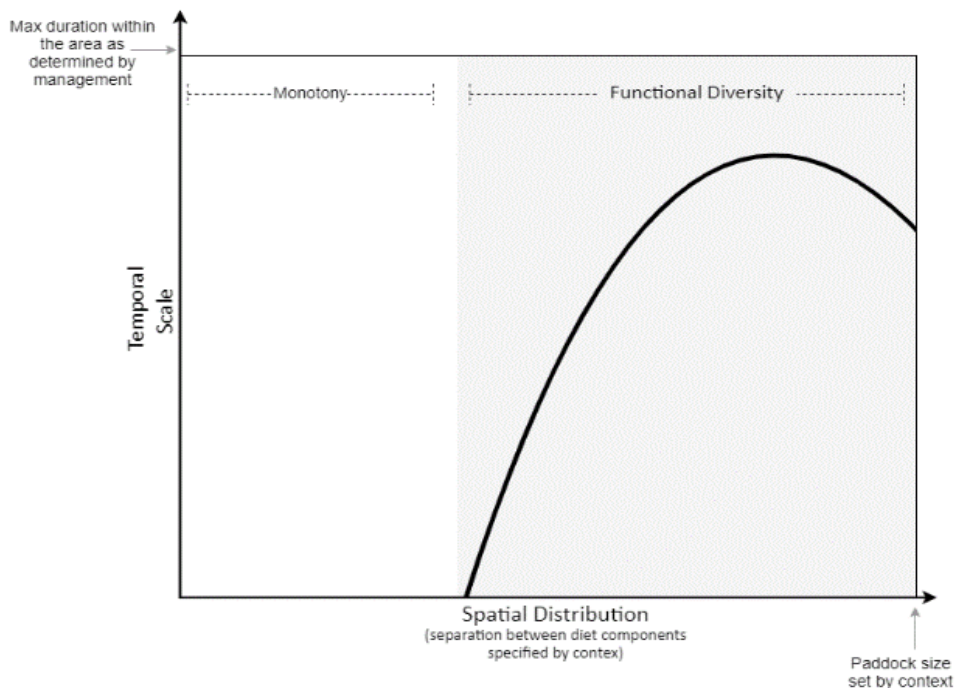
feed components may better meet animal requirements, or provide a beneficial complementary or synergistic effect upon consumption. Similar to dietary diversity, a diet can be functional in one context (time scale or component [as affected by spatial scale and separation] or animal), but monotonous at another (see Figure 2.2). Therefore, the optimal functionality of a diet is context specific for a particular time, space, and animal, and the relativity of the spatial distribution to the temporal scale is important in determining the functionality of diversity (Wallis de Vries and Schippers, 1994; Prins and Langevelde, 2008; Gregorini et al., 2017) (See Figure 2.1). The context and the scales used to define it (temporal or spatial distribution) can be manipulated to address different questions and problems, e.g. smaller scales may allow for inferences of within meal grazing decisions, while larger scales may allow for inferences on a larger unit of time stages e.g. diurnal grazing patterns.



**Figure 2.1** A conceptual model depicting how the spatial distribution of feeds vs time scale can influence the functionality of a diverse diet within a given context [e.g. spatial scale (paddock limitations), environment, the abundance of feed types, species richness], animal [allometry, morphology and physiology, experience, internal state, ecology (e.g. grazer or browser)]. The animal's context (e.g. physiological and morphological) determines a minimal spatial distribution at which selectivity of diversity can occur and anything smaller than this minimal distribution results in monotony (biochemical and/or sensorial) at the intake level. Conversely, animal context can limit the largest possible spatial distribution (traversable limit) at which selectivity can occur within a given temporal scale. Further, the temporal scale limits maximal spatial distribution at which it is possible to acquire different feeds (traversable limit). The functionality of the diversity is dependent on the context and the spatial distribution and temporal scales. The intermediate band between monotones represents the spatial separation of diet components at which an animal can formulate a functionally diverse diet of unique feeds within the given temporal scale. While the band of monotony at the bottom of the figure is indicative that if the temporal allocation of a diet is short enough, an animal would be only able to acquire a monotonous diet. The bandwidth and its positioning within the spatial and temporal scales is context specific (e.g. animal type). Within this band an optimum functionality exists (achievable or not), as demonstrated by the apex of the curve, where an animal is best able to utilize the different feeds available, this is context specific and is continuously changing through time.



a)



b)

**Figure 2.2** Demonstration of how the context, in this case animal size [a) small animal b) large animal] can affect the positioning of the monotony and functional diversity bands and the shape of the optimal functional diversity curve within a specified spatial scale. The smaller animal, can acquire functional diversity at a smaller spatial distribution, while the larger animal requires a greater spatial distribution before it can acquire functional diversity.

### 2.3.1 Functional Diversity and Production

As opposed to monotonously fed simple or diverse mixtures with low functionality, functionally diverse diets can increase production. Compared to monoculture diets, diverse diets with increased species richness through the spatial and temporal distribution can benefit soil, plant, and animal function, thereby affecting productivity at a range of system levels (Tilman, 1999; Di Falco and Chavas, 2006; Hector and Bagchi, 2007; Rodríguez et al., 2007; Eisenhauer et al., 2018; Schaub et al., 2020), including product quality (Moloney et al., 2008; Van Vliet et al., 2021; Vasta et al., 2008; Vasta and Luciano, 2011) and profitability. For example, Venning et al. (2003) found average daily gain of lambs was 30% greater for animals offered choice between grass and clover pastures compared with those fed a mixture of the two. This effect, of increased production when offered choice rather than a mixture, has been reported in a number of studies using sheep (Cosgrove et al., 2003; Champion et al., 2004; Venning et al., 2004) and cows (Nuthall et al., 2000; Rutter et al., 2003; Solomon, 2010). These studies suggest that functionally diverse pastures can improve the quality of the ingested diet on a per animal basis, providing evidence that how dietary components are presented can affect performance.

Improved production has been reported from dairy heifers (Handcock et al., 2015), bull calves (Pettigrew et al., 2017), sheep (Fraser et al., 1999; Golding et al., 2011; Somasiri et al., 2015; Grace et al., 2016; Grace et al., 2019; Jerrentrup et al., 2020), and goats (Murney et al., 2019) offered low functionality (low spatial separation) mixed pasture diets with high component richness compared to monoculture diets. For example, Golding et al. (2011) found that average daily gain of lambs offered a mixed pasture of plantain, chicory, red and white clover was 108% greater than those grazing ryegrass clover swards. The grass diets had a greater NDF, lower organic matter digestibility, and lower metabolizable energy than the diverse diets which the authors stated may have accounted for some of the production differences seen within this study, alongside grazing preference. However, results from mixed pastures with low functionality have been highly inconsistent with many studies reporting no production difference between such diets and those grazing simple or single species swards in dairy heifers (Handcock et al., 2015) and cows (Soder et al., 2006; Woodward et al., 2013; Edwards et al., 2015), sheep (Grace et al., 2016; Grace et al., 2019), and cattle (Jerrentrup et al., 2020). For example, Edwards et al. (2015) compared milk production of dairy cows grazing a ryegrass and white clover pasture with those grazing a diverse mixed pasture (ryegrass, white clover, plantain, chicory, and alfalfa) and found no difference in production. The same level of production reported by Edwards et al. (2015) occurred at the same level of DMI, however animals eating the diverse diet ingested less CP. The literature which showed increased



production on mixed swards used stock of smaller design (young stock or small species), thereby although the diet offered low functionality, the spatial separation of diets was perhaps great enough for the smaller animals to acquire functional diversity. The literature reporting no production differences from mixes typically used larger animals with reduced sorting capacity, thereby the diet may have presented them with low-functionality and intake would have been fairly monotonous. Further, greater production from functionally diverse diets is evidenced within the increased production reported by a number of studies from larger animals when they are provided diversity at a greater level of spatial separation (Nuthall et al., 2000; Cosgrove et al., 2001; Rutter et al., 2003; Champion et al., 2004; Marotti, 2004). For example, Cosgrove et al. (2001) saw an 11% increase in milk production from cows grazing spatially separated strips of ryegrass and white clover compared with those offered the same feeds as a mixture. Thereby, animal size within a given context may affect the functionality of the diet. This highlights that functionally diverse diets can perhaps provide benefits not possible when allocated dietary monotony and that there is a need to evolve our pastoral livestock production systems beyond the one-size-fits-all approach commonly practiced.

In addition to altering the spatial arrangement of feeds, the functionality of a diverse diet can also be manipulated by altering the temporal distribution of feed presentation. For example, Rutter et al. (2001) demonstrated that temporal distribution of feeds could yield the same milk production as cows offered free choice from the same feeds. Altering the temporal arrangements of feeds has also been shown to increase intake and production of both sheep (Papachristou et al., 2007; Lyman et al., 2008; Mote et al., 2008; Jensen et al., 2013) and cattle (Lyman et al., 2011) compared with those offered dietary monotony. For example, sheep consumed twice as much feed containing ergotamine D tartrate if it was allocated in sequence with tannins compared with when it was fed alone (Jensen et al., 2013). Another example of temporal feed allocation can be seen in the MENU model presented by Meuret and Provenza (2015), in where French herders design daily feeding circuits to stimulate feeding motivation and performance in target areas, forages or swards based on forage relative abundance and palatability to sequence meals in time and space. However, compared with research on the spatial arrangement of feeds, research regarding the temporal distribution of feeds (e.g. feeding sequences), particularly using fresh forages, is fairly scarce. Although studies have mainly used concentrates or silages, they provide credence to the theory that the functionality of a diet, through temporal distribution, can also provide production benefits compared with monotonous allocation through time.

Functionally diverse diets may increase intake and productivity as animals can customize diets through time to meet specific nutritional needs (Westoby, 1974), increase feed conversion

efficiency (Champion et al., 1998; Atwood et al., 2001; Atwood et al., 2006), avoid toxicosis (Freeland and Janzen, 1974), and increase motivation to eat through reduced habituation to specific nutrient or sensorial component of the diet (Early and Provenza, 1998; Epstein et al., 2009; Ginane et al., 2015), or perhaps a combination of all. However, not all experiments have reported increased intake, feed conversion efficiencies, or production with dietary choice (Şahin et al., 2003; Moya et al., 2011). For example, Şahin et al. (2003) reported no difference in intake, live weight gain, or feed conversion efficiency between Awassi lambs offered a total mixed ration (TMR) or choice from TMR diet components. Lack of response may arise where differences between feeds are insufficient in terms of providing a biologically significant difference in biochemical or sensorial properties for animals to meet their nutritional requirements or to stimulate the motivation to eat through alteration of sensorial input (Beck and Gregorini, 2020). These contrasting findings add evidence to our model that not only do feed types need to offer richness, but that they need to provide a biological meaningful divergence in biochemical or sensorial experience to provide functional diversity and the associated benefits to production.

Increased production as a result of diverse swards is not limited to benefits at the animal level, as mixed swards can also increase herbage yield (Tilman et al., 1996; Hector et al., 1999; Haughey et al., 2018) and temporal stability of yields after environmental, seasonal, and climatic changes (Tilman et al., 2001; Isbell et al., 2017; Haughey et al., 2018), although the spatial distribution to achieve this may not provide functional benefits for animals. Temporal stability of yields is a key limitation of simple swards (e.g. ryegrass white-clover) (Rawnsley et al., 2013). Although, a mixed sward may provide low functionality for the animal, it may be more functional for the plant in its dietary context, in terms of species facilitating the presence of another by changing the growing environment (e.g. soil structure) or increasing nutrient availability (e.g. legumes fixing N to allow for pasture growth) (Barry et al., 2019; Delory et al., 2019; Sonkoly et al., 2019). As such there is a tradeoff when planting diverse swards between the functionality of the spatial arrangement for plant growth and production and the functionality for the animal consuming it within the given context. Further, mixed swards provide a management dilemma in themselves, with more difficulties of pasture management (e.g. variable defoliation requirements from different species within the same sward) and weed management (e.g. no blanket spray regimes) compared with homogenous single or dual species sward or pastures of the same richness with spatial separation. Consequently, future research evaluating the production tradeoff between the functionality of the spatial and temporal arrangement for plant growth and animal production is required.

In summary, functional diversity can increase performance compared with a single feed or a diverse mixture. In addition, these benefits to animal production highlight the benefits of offering dietary diversity compared to a monotonous one-size-fits-all dietary approach.

### **2.3.2 Functional diversity: Animal Welfare**

Functionally diverse diets can enhance animal welfare compared with simple or diverse mixtures with low functionality fed repetitively. If diversity is provided functionally, animals can customize their nutrient intake, avoid or mitigate the toxic effects associated with over consumption of one nutrient (Freeland and Janzen, 1974; Westoby, 1974), and consume the correct dose of a plant [namely its plant secondary compounds (PSC)] to improve their antioxidant status, health, and consequently their hedonic welfare (Villalba and Provenza, 2007; Provenza et al., 2015; Beck and Gregorini, 2020). The antioxidant abilities of PSC reduce oxidative stress (Beck and Gregorini, 2020; Beck and Gregorini, 2021), which is thought to act in a mutual reinforcement cycle with physiological stress, where the presence of one elevates the other (Beck and Gregorini, 2020). As such, there is an association between oxidative stress and disease (e.g. mastitis) in livestock (Celi, 2010). Thereby, increased antioxidant status as achieved by a functionally diverse diet could be indicative of increased health and welfare (Manteca et al., 2008; Beck and Gregorini, 2021). In addition, ruminants offered functional diversity can self-medicate by ingesting the appropriate dose of a PSC (Villalba and Provenza, 2007; Villalba et al., 2011b; Provenza et al., 2015). For example, Villalba et al. (2006), observed that lambs fed, grain, tannins, and oxalic acid, in toxic quantities could select dose-appropriate quantities of three different 'medicinal' substances (sodium bentonite, polyethylene glycol, and dicalcium phosphate) to counteract the aversive effects of toxins. Thereby, functionally diverse diets can allow animals to acquire dose-dependent effects that would be unattainable to animals offered a monoculture sward or be unlikely to occur by chance of resulting species abundances within a mixture, or that would incur a greater detoxification cost. If animals are offered a functionally diverse diet and can self-medicate through the availability of an appropriate range of plants for a given context, that may reduce the need for laborious and costly animal intervention events, providing another economic benefit to functionally diverse diets.

In addition to improvements to hedonic well-being, effects of animals offered functionally diverse diets are thought to exhibit greater eudaimonic wellbeing (the pursuit and individual choices to acquire a good life and fulfill ones purpose) (Ginane et al., 2015; Beck and Gregorini, 2020) than those grazing simple or mixed diets. Eudaimonic wellbeing is thought to be improved through choice from diverse diet options as animals can reduce boredom and exert some control over their environment and express individuality [see Ginane et al. (2015) and Beck and Gregorini (2020)].

Evidence for such theories exist through the results of Ross (2006), who found captive polar bears offered choice between a holding space or an enclosure exhibited stress behaviors ~50% less frequently and increased play compared with those restricted to one area. Further, Catanese et al. (2013) observed a 37% reduction in blood cortisol concentrations [a common measure of welfare, see Barrell (2019)] from animals offered a choice between dietary constituents compared with those offered a TMR. This provides further evidence that welfare may be improved when a diverse diet is offered in a functional manner through spatial separation of diet components. However, no information is available on the effects of the temporal dimension of dietary diversity on animal welfare. We hypothesis that varying the functionality of a diverse diet through time also improves eudaimonic wellbeing by allowing animals to make decisions based on their current emotional, developmental, physiological, and morpho-physiological state. Future research is required into the effect on welfare of altering the temporal distribution of feeds, and offering a functionally diverse fresh forage diet, as a majority of the research to date has been done with grain, silage, or concentrate options.

In summary, functionally diverse diets may improve hedonic and eudiamonic wellbeing through the provision of choice, a strategy not available or limited to animals offered monocultures or mixed swards.

### **2.3.3 Functional diversity: Environmental Impact**

There is considerable societal pressure to reduce the negative environmental footprint of livestock systems, particularly in terms of greenhouse gas emissions and urinary N excretion (Gregorini et al., 2017). Reducing greenhouse gases is of particular interest as livestock production contributes to 7-18% of global anthropogenic greenhouse gas emissions (Stanley et al., 2018) that contribute to global warming (Beukes et al., 2010; Gerber et al., 2013). While high levels of nitrogen (N) emitted are another concern as N is readily volatilized to the greenhouse gas nitrous oxide (N<sub>2</sub>O) or leached into waterways, high levels of N in water have been linked to eutrophication (Cameron et al., 2013) and have even been linked to detrimental human health outcomes [e.g. cancer and blue baby syndrome (Schullehner et al., 2018; Fossen Johnson, 2019)]. Diverse and Functionally diverse pastures (spatial and temporal variation) can reduce environmental impacts of livestock compared to simple or single component diets and they can also increase animal performance (Rodríguez et al., 2007; Al-Marashdeh et al., 2020; Beck and Gregorini, 2021), increasing feed conversion efficiency (FCE)(Waghorn and Hegarty, 2011; Al-Marashdeh et al., 2020; Beck and Gregorini, 2021), greater utilization or sequestration of excreted nutrients by plants (Pembleton et al., 2015; Vibart et al., 2016; Whitehead et al., 2018), and through the positive actions of some PSC (Hess et al., 2003;

Zhang et al., 2019; Ku-Vera et al., 2020). Although, we are unable to make a blanket statement as to how functional diversity will reduce environmental impacts compared with monotony, as each case is context specific, this review highlights functional diversity as a promising tool that requires future research.

As mentioned within the production section, functionally diverse diets are often more effective than monotonous single species diets or non-functionally diverse diets at improving performance. Such improvements in livestock performance are associated with reductions in the emission intensity (pollutant per kg of product) and a reduced number of days to reach slaughter weight (Beck and Gregorini, 2021). Extrapolating data from diets of differing functionality from Venning et al. (2003), lambs (starting live weight 18 kg) could have been finished (~40 kg) on the mixed ryegrass and clover diet after 79 days or 61 days if provided the feeds in a functional manner, indicating that days to slaughter could be reduced by 23%. However, further studies are needed to explore this. Further, offering animals diverse diets or choice to formulate their diets increases FCE (i.e. nutrient use efficiency) compared with those grazing a more monotonous diet (Nocek et al., 1986; Villalba et al., 2011a; Al-Marashdeh et al., 2020), resulting in less feed required per animal and/or reduced days to slaughter, and reduced emissions (e.g. nitrogen and methane). For example, Al-Marashdeh et al. (2020) reported a greater FCE and N retention for lambs grazing a diverse mixed sward compared with those grazing a simple sward, indicating that diverse lambs could meet target weight in a shorter time with less N lost to the environment. Based on this literature we would expect that functional diversity would increase FCE and thereby would reduce environmental impacts in terms of both reduced urinary N and methane. The results of these works provide evidence that functionally diverse diets can reduce the environmental impacts of livestock production systems.

Negative environmental impacts can also be reduced through the actions of PSC that animals ingests (Hess et al., 2003; Zhang et al., 2019; Ku-Vera et al., 2020). Functionally diverse diets can strategically incorporate known quantities of plants containing environmentally beneficial PSC into animals diets, eliminating 'chance dosing' and discrepancies in the proportions of plants in a less-functional mixture, or by providing the diet in a functional manner that allow animals to select an appropriate dose themselves. For example, plantain is a herb rich in the PSCs aucubin and aetoside, which are known to reduce ammonia production by 40% compared with chicory (which does not contain these PSC) (Navarrete et al., 2016), and would reduce N loss (urinary N) (Attwood et al., 1998). However, when sown within a mix, plantain rarely exceeds 20% of available pasture (Muir, 2011), and is required to comprise  $\geq 30\%$  of the diet to reduce urinary N excretion

(Nkomboni, 2017). These findings highlight how low functionality mixes can lack environmental benefits that can be generated when the diet is offered functionally, and set areas of different species are allocated. Different plants contain a large range of PSC (with direct and indirect actions) (Zhang et al., 2019; Ku-Vera et al., 2020) that can reduce methane and nitrogen excretion and could be incorporated in functionally diverse diets to reduce environmental impact. Thereby, considering the actions of PSC alongside the earlier mentioned increases in FCE and changes in ingestion and digestion dynamics when allocated a functionally diverse diet, we also expect differences in rumen function and throughput from animals fed such a diet.

Further, strategic pairings of feeds within functional diets can provide complementary relationships between ingested PSC, which can reduce environmental impacts (Tilman, 1982; Villalba et al., 2019; Lagrange et al., 2020). For example, Lagrange et al. (2020) found that heifers offered a choice between 3 tanniferous feeds (birdsfoot trefoil, sainfoin, and alfalfa) retained more nitrogen and partitioned less into urine compared with those grazing monocultures. As such, providing a functionally diverse diet can incorporate specific quantities of plants into animals diets, reducing the 'chance-dosing' that can occur within less-functional mixtures on pastures where selective foraging and competition among plants alters availability of plants. Further, the incorporation of such species within a diverse diet could allow for production, health, welfare and environmental benefits to be utilized simultaneously.

Mixed swards with low functionality can better utilize nutrients, including those within or excreted into the soil (Sanderson et al., 2004; Pembleton et al., 2015; Vibart et al., 2016). One example of greater environmental benefit from diverse swards was seen by McNally et al. (2015), who found that a diverse mixed-pasture (ryegrass, white clover, prairie grass, chicory, plantain, and alfalfa) put an estimated 1203 kg per ha per year more carbon into the top 300 mm of soil than a ryegrass-clover pasture and hypothesized this was due to increased root mass inputs and rooting depth. Mechanisms of action for reduced soil nitrate levels were hypothesized by Sanderson et al. (2004) as complementary actions of different species, less soil N mineralization, or through the high N uptake of a deep rooted species. However not all results have yielded positive reductions in N leaching, with Hooper and Vitousek (1998) finding no effects of increasing plant diversity on nitrates in the soil below the rooting zone. These inconsistencies could be due to different plant species and soil types between studies. However, such mixed pastures provide management challenges (i.e. determining an appropriate defoliation height that suites all species) and in planting as that can reduce the functionality of the diet at the animal level.

In summary, these studies imply that increases in production, feed (i.e. nutrient) use efficiency, and greater utilization of PSC and their complementary actions acquired through a functionally diverse diet can reduce the impacts of ruminant production on the environment.

## **2.4 Functional diversity in the context of diet composition and presentation, and animal design**

The wide-ranging ramifications that the functionality of a diet can have within livestock systems leads to the question of how diverse diets might be designed? And what are the tools we can use to design them? The conceptual model developed in Figure 7.3 indicates the levels at which we can affect the functionality of the diet within systems in a given environment. These are through the i) context, ii) spatial distribution, and iii) temporal distribution, and the tools with which we can use to manipulate each i-iii) and the relationships between these levels and tools. The levels at which the functionality of diversity can be affected by tools are outlined in Figure 7.1, temporal scale (y-axis), spatial distribution (x-axis), and the size and placing of the band width and optimal functionality as determined by the context.





Temporal variation can be used to alter the temporal scale at which diversity is provided. Tools for manipulating the temporal variation include frequency of paddock rotation, altering the sequence of feed presentation, both of which are also impacted by determinants of the spatial scale (e.g. paddock size and animal number). These temporal tools can be used to design feed combinations and determine presentation of diets. An example of this MENU model presented by Meuret & Provenza (2015), in where French herders design daily feeding circuits to stimulate feeding motivation in target areas, forages or swards based on forage relative abundance and palatability to sequence meals in time and space. In addition, the context within a given environment that functionality of diversity can be influenced by management include the richness of the diet available, the abundance of each feed component, feed combinations, the animal, its species, the number of animals within a herd, paddock size, and its individuality. Management can influence individuality, through genetic selection, manipulating its past experiences (e.g. *in utero* and early life programming), dictating its nutritional state, current health, and welfare, all of which flow into each other and the behavior or personality of a given animal. Furthering the complex relationships between richness, abundance, and feed combinations is how they are distributed through space (planting arrangement), which will also impact the temporal scale at which an animal can acquire diversity (see Figure 2.1). The importance of each of these levels of management on the system (production and product quality, profitability, animal health and welfare, and the environmental impact) require further investigation as we have only just scratched the surface of the potential of improving production, product quality, health, and welfare, and reducing the environmental footprint of our systems through functional diversity.

A contextual example of altering functional system design could be the requirements of different levels of spatial separation for grazing cattle compared with sheep (Similar to the example given in Figure 2.2 of a small vs large animal). For example, based on the morpho-physiological and allometric differences alone, sheep could graze selectively from pastures differentiated by a smaller scale than cows. This is evidenced by Hilario et al. (2017) who reported no difference in grazing behavior (time walking, ruminating, or grazing) of cows grazing rich-mixed swards or a grass-dominated sward, whereas sheep, in agreement with Champion et al. (2004), tended to spend more time walking and spent 6.4% more time ruminating, and 6.7% less time grazing while on the mixed-sward, indicating sheep were better able to graze selectively in these swards. Another example of this is provided in how smaller species such as goats and sheep are more likely to consume browse species than cattle, as they can sort high quality parts such as young growth, flowers and pods (Rook et al., 2004). If such an experiment was repeated with spatially separated pastures we might expect a difference in the grazing behaviors of cattle on the diverse diet due to its provision in a functional manner. Research is required into the exact size or area 'optimal' for each animal for set diet

richness. Another potential difference in functional diet design between sheep and cattle, highlighted by Orr et al. (1996) and Hilario et al. (2017), is the temporal dimension at which grazing events occur. Hilario et al. (2017) indicated that cattle, which generally have three main meals in a day (Gregorini, 2012), spent longer ruminating and resting after grazing than sheep, which alternate short grazing and ruminating bouts (Hilario et al., 2017), which matches Orr et al. (1996) hypothesis that cows required longer ruminating as a result of less processing during ingestion. Thereby, sequence of feed presentation to cows may be most effective by matching feed supply to meals, whereas sheep may benefit from more regular alteration to feed presentation. How the functionality of diverse diets can be best designed within different contexts requires further investigation. The conceptual model (Figure 2.3) outlines some of the tools that could be used to design pastures and areas in which future research is required.

Of concern regarding design of diverse diets is that animals often display neophobia [fear of novel situations and stimuli (Crane et al., 2020)] and strong preference towards familiar species. Whilst, neophobic behaviors have a biological purpose and are maintained throughout an animals life (Launchbaugh and Provenza, 1991), allowing ruminants to learn and distinguish between foods that meet nutritional requirements or provide beneficial properties and avoid those that are nutritionally poor or toxic (Provenza and Balph, 1988; Launchbaugh and Provenza, 1991), if neophobia is too strong it can provide its own issues to systems as animals may not utilize plants strategically incorporated into systems or overgraze familiar feeds. Overgrazing of familiar feeds can elevate the time spent and damage (e.g. pugging) in areas containing such feeds, while areas containing non-familiar feeds may remain underutilized despite having been included in feed budgets (Launchbaugh and Howery, 2005; Beck and Gregorini, 2021). As such, neophobic behaviors may inhibit the uptake and utilization of diverse diets as they would incur financial, productivity, and environmental challenges of their own which would potentially outweigh those within simple monotonous system designs. Dietary exposure and experience can reduce neophobia. Experience while the animal is more plastic, in *in utero* (Hai et al., 2013), early life (Langlands, 1967; Schaal et al., 1995; Hai et al., 2013; Beck et al., 2021), through milk ingestion (Provenza and Balph, 1988; Nolte and Provenza, 1991; Mennella et al., 2009; Wenning and DeLiberto, 2010), and learning from experience (Provenza, 1996; Villalba et al., 2015a) from peers or dams, can alter animals preferences, reduce neophobia and may alter emotions (interpretation of a cluster of experiences) (Beck and Gregorini, 2020; Beck and Gregorini, 2021). This learning includes social experiences, with wisdom imparted by mothers having a greater influence on intake by offspring than that of other peers. For example, Thorhallsdottir et al. (1990) found lambs offered novel foods alongside the dam will consume twice as much compared with lambs exposed to the feed alongside unfamiliar ewes. These dietary experiences in early life have a greater influence on shaping preference in later life than

experience as an adult (Provenza and Balph, 1988). Thereby, management and supply of a diverse and varied diet during this 'early life' period could teach animals to utilize their environment more uniformly and to consume plants with beneficial properties (e.g. medicinal), which could reduce the need for laborious and costly intervention practices regarding health (e.g. worming), thereby reducing the workload. As such, the design of systems to include functionality are not only designing future landscapes but potentially the future animals that are grazing within them.

## 2.5 Concluding remarks

The overlap between diverse and monotonous diets, as they are part of the same continuum of possible feed combinations and spatio-temporal arrangements, has driven the need for a descriptor of this continuum and diets that are more successful at meeting an animal's individual nutritional needs through allowing preference behaviors. We have coined this descriptor as functional diversity. Evidence for the benefits of functionally diverse diets over non-functionally diverse diets can be seen in animal production, health, welfare, and environmental improvements where animals were presented with more-functional choice and variation through time, thereby consuming a more 'optimal' intake for their individual needs, compared to those offered the same diet components in a monotonous manner. Moreover, evidence for functional diversity shows it is not just the nutritional composition of the diverse diet, but also how it is presented to the animal that can impact the system at a range of levels. Key areas requiring further work, highlighted by this review, include: the scale (temporal and spatial distribution) at which each of these tools needs to be utilized to have an impact, what level of species richness and abundance is required to provide a functionally diverse diet or if the exposure can be varied to provide maximal experience to a range of plants, and the degree of influence that early life experience has to elicit recognition and incorporation of different foods in later life.

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## Chapter 3

# The Effect of Incorporating Plantain, Chicory, and Alfalfa, into a Ryegrass-based diet on *In Vitro* Gas Production and Fermentation Characteristics

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### 3.1 Abstract

**Objective:** The objective of these experiments was to determine how altering the proportion of ryegrass (*Lolium perenne* L.) to chicory (*Cichorium intybus* L.), alfalfa (*Medicago sativa* L.), or plantain (*Plantago lanceolata* L.), or a mixture (equal parts DM of chicory, alfalfa, and plantain) affects the *in vitro* fermentation rates and formation of fermentation products.

**Materials and Methods:** *In vitro* experiments were conducted using the ANKOM<sup>RF</sup> gas production modules (ANKOM, Macedon, NY, USA). Experiment 1 examined increasing inclusion level (0, 50, 100%) of chicory, plantain, or alfalfa with ryegrass and experiment 2 examined increasing inclusion level (0, 25, 50, 75, 100%) of ryegrass to a mixture (equal parts DM chicory, plantain, and alfalfa).

**Results and Discussion:** In experiment 1, there was a positive linear effect for 24 hr gas production per g DM as inclusion increased for chicory ( $P < 0.01$ ) and plantain ( $P < 0.01$ ). A negative linear relationship was detected for ammonia (NH<sub>3</sub>) and branched-chain volatile fatty acids (VFA) as levels of plantain ( $P < 0.01$ ) and chicory ( $P < 0.01$ ) increased. In experiment 2, there were linear ( $P < 0.01$ ), quadratic ( $P < 0.01$ ), and cubic ( $P < 0.01$ ) effects on both the asymptote of the gas production curve (ml/g DM) and the fractional rate of gas production (%/hr) with increasing mixture inclusion. There was a linear decrease in the total VFA comprised of iso-butyrate ( $P < 0.01$ ), and iso-valerate ( $P < 0.01$ ) as the level of chicory, plantain, and alfalfa inclusion increased.

**Implications and Applications:** Incorporating greater levels of chicory, plantain, or a mixture of chicory, plantain, and alfalfa, with ryegrass increased gas production and reduced NH<sub>3</sub> and branched-chain VFA in rumen fluid, which implies a greater nutrient supply from the rumen to the host animal. Further research is required to evaluate whether these results translate to *in vivo* benefits on animal performance and reductions of urinary N excretion.

**Key words:** *in vitro*, rumen fermentation, diverse diet, ryegrass

### 3.2 Introduction

New Zealand pastoral grazing systems are predominately perennial ryegrass (*Lolium perenne*) and white clover (*Trifolium repens*) –based, as these palatable forages provide high yields in a range of temperate environments and grazing management systems (Carlson et al., 1996; Delagarde et al., 2000). Ryegrass-white clover swards often have greater concentrations of crude protein (CP) and smaller concentrations of water soluble carbohydrates (WSC) and are known to oversupply nitrogen (N) to grazing livestock, resulting in inefficient N utilization (Edwards et al., 2007; Gregorini et al., 2016). Inefficient N utilization, increases ruminal ammonia (NH<sub>3</sub>) and urinary N excretion, which in turn exacerbates N lost from the system through nitrate leaching and nitrous oxide (N<sub>2</sub>O) emissions (Di and Cameron, 2002; Edwards et al., 2007), and causes food aversions in ruminants (Provenza 1995). Other disadvantages of ryegrass-white clover swards include reduced yield over dry summers (Pembleton et al., 2015), dwindling clover persistence over time (Brock, 1988), and pest challenges [e.g. black beetle (*Heteronychus arator*) and grass grub (*Costelytra zealandica*)] (Zydenbos et al., 2011; Woodward et al., 2013). These disadvantages have led to increased interest in incorporating alternative forage species into ryegrass-based swards and grazing systems. Further, diverse diets may improve animal health (Provenza et al., 2007; Dixon and Pasinetti, 2010), and productivity (Golding et al., 2011; Garrett et al., 2021) while reducing the environmental impact of grazing animals (Flores, 2020; Garrett et al., 2021). Chicory (*Cichorium intybus* L.), plantain (*Plantago lanceolata* L.), and alfalfa (*Medicago sativa* L.) are examples of alternative species incorporated into monocultures to bolster production and feeding value to animals (Brown et al., 2000; Burke et al., 2002; Moorhead et al., 2002; Lee et al., 2015). Compared to ryegrass swards, diverse swards that contain both legumes (e.g. alfalfa) and herbs (e.g. chicory and plantain) can supply equivalent or greater dry matter (DM) production and a greater metabolizable energy (ME) production per ha per year (Nobilly et al., 2013) and can increase animal production (Golding et al., 2011). Currently, there is a paucity of information on the effects of either herbs or legumes as individual diet components, in binary mixes, or as part of complex sward mixture on rumen fermentation. Therefore, the objective of this study was to determine how altering the proportion of ryegrass with either chicory, alfalfa, or plantain, or a

mixture of equal parts chicory, alfalfa, and plantain affected the *in vitro* formation of ruminal fermentation products — an indicator of fermentability and nutrient supply from the rumen.

### **3.3 Materials and Methods**

All procedures were approved by the Lincoln University Animal Ethics Committee (AEC 2019-28).

#### **3.3.1 Experimental Design**

##### **3.3.2 Experiment 1.**

Substrates of different proportions (0, 50, or 100%) of alfalfa (cv. Titan), chicory (cv. Choice), or plantain (cv. Agritonic), with perennial ryegrass (cv. One<sup>50</sup>) were examined and the effects on rumen fluid fermentation and gas production were compared using the ANKOM<sup>RF</sup> Gas Production Systems (ANKOM, Macedon, NY, USA). The experiment was comprised of three runs (24 hr per run) with jar considered as the replicate. Within each run every treatment was replicated twice (two fermentation jars per run). Additionally, each run contained three control jars of 100% ryegrass. Treatment to jar allocation was randomized for each run.

##### **3.3.3 Experiment 2.**

Substrates of different proportions (0, 25, 50, 75, or 100%) of ryegrass with the remaining substrate proportion comprised of equal parts chicory, alfalfa, and plantain. The effects on rumen fermentation and gas production were compared within ANKOM<sup>RF</sup> Gas Production Systems (ANKOM, Macedon, NY, USA). Experiment 2 was comprised of three 24 hr runs, with each treatment replicated in triplicate for each run (three fermentation jars per run). Treatment to jar allocation was randomized for each run.

##### **3.3.4 Animal Handling and In Vitro Fermentation.**

One liter of ruminal fluid was collected from non-lactating rumen-cannulated cows (Holstein-Friesian and Jersey cross; live weight = ~600 kg; n =4) grazing perennial ryegrass supplemented with alfalfa silage at Ashley Dene Sheep Research farm. Rumen fluid was collected by taking rumen digesta from random locations within the rumen and straining it through cheesecloth into a warmed (39.5°C) thermos flask and purged with carbon dioxide (CO<sub>2</sub>) to maintain anaerobic conditions.

Rumen fluid was re-strained through cheese cloth and subsampled (20 ml per jar) into fermentation jars containing 80 ml of buffer solution. Buffer solution was prepared according to the operating instructions of ANKOM (2018) and consisted of combining two warmed (39.5°C) solutions: buffer A (KH<sub>2</sub>PO<sub>4</sub> at 10 g/L, MgSO<sub>4</sub>•7 H<sub>2</sub>O at 0.5 g/L, NaCl at 0.5 g/L, CaCl<sub>2</sub>•2 H<sub>2</sub>O at 0.1 g/L, and reagent grade urea at 0.5 g/L) and buffer B (Na<sub>2</sub>CO<sub>3</sub> at 15.0 g/L and Na<sub>2</sub>S•9 H<sub>2</sub>O at 1.0 g/L) at an 5:1

ratio, adjusted to a pH of 6.8. Each fermentation jar contained 1.00 g DM of substrate (corrected for residual DM), as per the randomized treatment to jar allocation for each run. Throughout the loading of fermentation jars, the mixture of rumen fluid and buffer medium were maintained at 39.5°C within a water bath and purged with CO<sub>2</sub>. The loaded ANKOM jars, fitted with the ANKOM<sup>RF</sup> Gas Production System (ANKOM, Macedon, NY, USA), were placed within an oscillating incubator set at 60 rpm (Minitron, INFORS HT, Switzerland) for 24 hr. The ANKOM<sup>RF</sup> Gas Production System automatically records the gas pressure and temperature every 5 min over the 24 hr period. At the termination of the 24 hr gas production period the pH of the fluid was measured using a benchtop pH meter (Orion 2-star, Thermo Scientific, Beverly, USA). The non-degraded substrate was filtered into Dacron bags (Custom Advanced Connections; 10 × 15 cm with 50-µm pore size), which as per Mehrez and Ørskov (1977) were rinsed with cold water and dried at 60°C for 48 hr to calculate apparent dry matter digestibility (ADMD). Subsamples of the rumen fluid-buffer solution and substrate were collected within 2 ml Eppendorf tubes, to determine volatile fatty acids (VFA) concentration and another sub-sample was acidified (10 µl of 99% H<sub>2</sub>SO<sub>4</sub>) to determine NH<sub>3</sub>. These samples were stored at -20°C until analysis.

### 3.3.5 Forage Sampling and Preparation.

Herbage was cut approximately 3 cm above ground level with a Haldrup forage harvester (Haldrup GmbH, Ilshofen, Germany). Ryegrass, chicory, and alfalfa were all in a vegetative state, while plantain contained 35% DM reproductive stem and seed head. The chicory was first year with a standing height of 35 cm prior to harvesting, ryegrass was at the three leaf stage of growth, and alfalfa was at a late vegetative state (Hall, 1996). Herbage used as substrate was frozen (-20°C), freeze dried and then ground by a centrifugal mill (ZM200; Retsch, Haan, Germany) to pass through a 1mm screen.

### 3.3.6 Laboratory Analysis.

The chemical composition of herbage (Table 1) was determined using near infrared spectroscopy (NIRS; Model: FOSS NIRS Systems 5000, Maryland, USA). Herbage metabolizable energy (ME) was estimated using the Primary Industries Standing Committee, (2007) equation:

$$[ME (MJ/kg DM) = \text{digestible OM in DM, \%}(DOMD) \times 0.16] \quad [1].$$

The NH<sub>3</sub> concentration of the acidified rumen samples was measured using a clinical analyzer (Randox Rx Daytona, Crumlin, Co. Antrim, UK) and a commercial test kit (Cat. No. AM3979; Randox; Crumlin, Co. Antrim, UK) based on the enzymatic UV method described by Neeley and Phillipson (1988). The VFA concentrations within non-acidified rumen samples was determined using a Gas

Chromatograph (GC: Shimadzu GC-2010, Kyoto, Japan with AOC-20i auto-sampler) fitted with a SGE BP21 30 m × 530 µm × 1 µm wide-bore capillary column as described by Chen and Lifschitz (1989).

### 3.3.7 Statistical Analysis.

All data were analyzed using R (R Core Team, 2019, v.3.6.0). The experiments were a one-way factorial design. Gas production data were converted to ml gas produced from psi using the ideal gas law and Avogadro's number. Data were normally distributed and had homogeneous variance, thereby satisfying the assumptions of the model. Total gas production in both ml/g DM and ml/g OM, concentrations of VFAs, NH<sub>3</sub>, and pH were analysed using a mixed model analysis of variance (ANOVA) using the *lmer* function of the *lme4* package (Bates et al., 2015). Jar was the experimental unit, treatment, inclusion level, and the treatment×inclusion level interactions were the fixed effects and run was a random effect. Following a significant ANOVA in experiment 1, contrasts were generated to compare forage species within inclusion level and polynomial contrasts (linear, quadratic, and cubic) were generated to compare increasing inclusion level within forage species. In experiment 2, only polynomial contrasts were generated. All contrast were generated using the *emmeans* package (Lenth, 2018). The Ørskov and McDonald, (1979) model, i.e.,

$$p = b (1 - e^{-ct}) \quad [2].$$

was used to analyse the gas production data where: b is the theoretical asymptote of the gas curve, c is the fractional rate of gas production (%/hr), and p is the gas production after time 't'. The Ørskov and McDonald, (1979) model was fit using the *nlme* function (Pinheiro et al., 2019). Following fitting of the model, the effects of dietary treatments on the model parameters were first explored using the *anova.lme* function of the *nlme* package, which implements Walt tests for the model terms (Pinheiro et al., 2019). All figures were created using the *ggplot2* package of R (Wickham, 2016). To illustrate the gas production curves graphically, a generalized additive model (GAM) was plotted using the *ggplot2* package. Statistical significance was declared at  $P \leq 0.05$  with trends declared at  $0.05 < P \leq 0.10$ .

## 3.4 Results

### 3.4.1 Results Experiment 1.

Gas production over 24 hr at different levels of inclusion of chicory, plantain, and alfalfa with ryegrass are presented in Figure 3.1. Positive linear effects were detected for 24 hr gas production per g DM and per g of OM as inclusion increased for chicory ( $P < 0.01$ ; Table 3.2) and plantain ( $P < 0.01$ ). The asymptote of the gas production curve (parameter b, mL/g DM; Figure 3.3) at 50 and 100% inclusion where different between all treatments ( $P < 0.01$ ). Further, there was a linear effect on

parameter *b* as inclusion level increased within treatment for chicory ( $P < 0.01$ ), alfalfa ( $P < 0.01$ ), and plantain ( $P < 0.01$ ). Both linear and quadratic effects were detected for the fractional rate of gas production (parameter *c*, %/hr) as the level of inclusion for each treatment increased ( $P \leq 0.01$ ; Figure 3.3).

There was a linear increase and decrease in ADMD, for chicory and alfalfa respectively, as their level of inclusion increased ( $P < 0.01$ ; Table 3.2, Table 3.3). The ADMD of the 100% alfalfa substrate was 42% less digestible than the 100% chicory substrate and 37% less digestible than the plantain substrate ( $P < 0.05$ ), which were not different ( $P > 0.10$ ). A negative linear relationship was detected for  $\text{NH}_3$  and pH with increased level of inclusion of plantain ( $P < 0.01$ ) and chicory ( $P < 0.01$ ). There was a positive linear increase for total VFA with increasing chicory inclusion ( $P < 0.01$ ). There tended to be a linear decrease in the iso-butyrate ( $P = 0.10$ ) and iso-valerate ( $P = 0.10$ ) with the increase in inclusion of chicory, while there was a linear decrease in valerate, iso-butyrate, and iso-valerate with increasing plantain inclusion ( $P < 0.01$ ). There was a linear decrease in the proportion of total VFA concentration comprised of valerate as the level of inclusion increased for chicory ( $P < 0.01$ ), alfalfa ( $P = 0.02$ ), and plantain ( $P < 0.01$ ). In addition, there was a linear decrease in the proportion of total VFA comprised of branched-chain VFA, iso-butyrate and iso-valerate as the level of inclusion of chicory ( $P < 0.01$ ) and plantain ( $P < 0.01$ ) increased.

### 3.4.2 Results Experiment 2.

Gas production over 24 hr at different levels of ryegrass inclusion with remaining substrate comprised of equal parts chicory, plantain, and alfalfa are presented in Figure 3.2. A cubic effect was detected for both 24 hr gas production per g DM and per g OM across the different levels of ryegrass inclusion ( $P < 0.01$ ; Table 3.4). There were also linear ( $P < 0.01$ ), quadratic ( $P < 0.01$ ), and cubic ( $P < 0.01$ ) effects on both the asymptote of the gas production curve (parameter *b*, ml/g DM; Figure 3.4) and the fractional rate of gas production (parameter *c*, %/hr) with increasing ryegrass inclusion.

There were no linear, quadratic, or cubic effects for ADMD and total VFA concentration as the level of inclusion of ryegrass decreased ( $P > 0.10$ ; Table 3.4) but there was a linear decrease in pH ( $P < 0.01$ ),  $\text{NH}_3$  ( $P < 0.01$ ), valerate ( $P < 0.05$ ), and branched-chain VFA ( $P < 0.01$ ). There was a quadratic effect ( $P < 0.05$ ) and tendency ( $P = 0.10$ ) for a positive linear effect between the proportion of total VFA comprised of acetate and the level of chicory, plantain, and alfalfa. There were quadratic effects between the inclusion of chicory, plantain, and alfalfa and the proportion of total VFA comprised by propionate ( $P = 0.05$ ) and acetate ( $P = 0.02$ ). There was a linear decrease in the total VFA comprised of valerate ( $P < 0.01$ ) and hexanoate ( $P < 0.01$ ), iso-butyrate ( $P < 0.01$ ), and iso-valerate ( $P < 0.01$ ) as the level of chicory, plantain, and alfalfa inclusion increased.



### 3.5 Discussion

The linear increase in gas production relative to the ryegrass only substrate with increased inclusion of chicory, plantain, and the mixture (equal parts chicory, plantain, and alfalfa) are likely the result of differences in substrate digestibility and chemical composition, namely the greater ADMD and WSC, and lower CP content of substrates. Apparent degradability and gas volume have been reported by Blümmel et al. (1997) to be strongly correlated and an increased availability of ruminal fermentable energy (e.g. WSC) can increase ruminal microbial protein synthesis (Krause et al., 2002). Differences in dietary protein intake (lower CP with greater chicory, plantain, and mixture inclusion) may explain some of the differences in branched-chain VFA and  $\text{NH}_3$  concentrations. Further the proportion of protein that is rumen un-degradable is greater for chicory and plantain compared to ryegrass (Minneé et al., 2018), thereby increased incorporation of chicory, plantain, and inclusion within the mixture may further exaggerate the dietary differences of protein available for proteolysis. Proteolytic bacteria, such as *Megasphaera elsdenii*, reduce branched-chain amino acids from protein substrate to branched-chain VFA and  $\text{NH}_3$ , therefore less precursor material (CP) would limit proteolytic production of branched-chain VFA and  $\text{NH}_3$ . In fact, dietary protein supply and ruminal branched-chain VFA and  $\text{NH}_3$  concentration can be positively correlated (El-Shazly, 1952; Annison, 1954; Beck et al., 2021). However, as  $\text{NH}_3$  and branched-chain VFA are not always the terminal end-product, their concentration alone is not always reflective of the extent of protein degradation (Apajalahti et al., 2019). Several strains of cellulolytic and non-cellulolytic bacteria utilize branched-chain VFA for growth (Allison et al., 1962b; Allison et al., 1962a), thereby low ruminal branched-chain VFA concentrations can be indicative of greater growth from such bacteria.

The decrease in CP:WSC ratio of diets with greater plantain, chicory, or mix inclusion may explain the linear decrease in ruminal  $\text{NH}_3$  and branched-chain VFA concentrations (Figure 3.5). Improvements to the rumen supply of energy can increase microbial protein synthesis and the efficiency of N use, and thereby reduce rumen  $\text{NH}_3$  (Miller et al., 2001; Krause et al., 2002; Vibart et al., 2009; Totty et al., 2013). This relationship exists as a larger amount of  $\text{NH}_3$  is utilized as the N source for microbial protein synthesis and carbohydrate availability is the primary limitation of  $\text{NH}_3$  utilization by rumen microbes (Navarrete et al., 2016). Increased energy availability paired with lower ruminal  $\text{NH}_3$  can be indicative of greater  $\text{NH}_3$  incorporation into microbial protein (Raab et al., 1983; Navarrete et al., 2016), but low  $\text{NH}_3$  can also be the result of inadequate dietary protein supply limiting microbial protein production (Raab et al., 1983). Greater gas production after 24 hr (Figure 3.1 and 3.2) and a greater asymptote (b parameter) of gas production from non-linear Ørskov model (Ørskov and MacDonald 1979), alongside reduced  $\text{NH}_3$  and branched-chain VFA concentrations, suggest greater microbial activity within the lower CP:WSC diets. Lower CP:WSC diets within the

present study were those which incorporated a greater proportion of plantain, chicory, or mix rather than insufficient protein supply.

Reductions in  $\text{NH}_3$  occurred with increased inclusion of chicory, plantain, and the mix (chicory, plantain, and alfalfa), likely due to the chemical properties of chicory and plantain. Plantain tended to ( $P = 0.10$ , at 50% inclusion) and did have lower ( $P < 0.05$ , at 100% inclusion) levels of  $\text{NH}_3$  than chicory in experiment 1, despite similar primary chemical composition (i.e. CP and WSC), as plantain has a greater level of rumen undegradable protein (Minneé et al., 2018). Some plant secondary compounds in plantain, such as aucubin and acteoside, reduce  $\text{NH}_3$  production through bactericide activity or by providing additional energy sources (Navarrete et al., 2016). While, chicory contains condensed tannins which bind to the proteins protecting them from rumen degradation and increase the N proportion excreted in the feces (Lagrange and Villalba, 2019). Reduced ruminal  $\text{NH}_3$  concentrations are associated with reduced N lost to the environment via urination (Attwood et al., 1998), therefore the reductions in  $\text{NH}_3$  production due to increased inclusion of chicory, plantain and a chicory, plantain, and alfalfa mix in this study could be indicative of a reduced environmental impact (e.g. through reductions in nitrate leaching and volatilization) from animals grazing these diets *in vivo*.

### **3.6 Applications**

In summary, incorporating greater levels of chicory, plantain, or a mixture of chicory, plantain, and alfalfa, with ryegrass increased gas production, indicating greater fermentation, and reduced ruminal  $\text{NH}_3$  and branched-chain VFA implying a potentially greater level of microbial protein synthesis and consequently nutrient supply to the host animal. The lower ruminal  $\text{NH}_3$  production *in vitro* may correspond to biologically significant reductions of urinary N excretion and nitrate leaching *in vivo*, this may be due to partitioning of more N into the feces, which in temperate is not the major cause of N leaching. Further *in vitro* and *in vivo* research is required to support these statements.

**Table 3.1** Chemical composition of substrates used within 24 hr fermentation within ANKOM<sup>RF</sup> Gas Production Systems.

Item <sup>2</sup>	Substrates <sup>1</sup>											
	Experiment 1						Experiment 2					
	0	50			100			Ryegrass				
		Chicory	Alfalfa	Plantain	Chicory	Alfalfa	Plantain	0	25	50	75	100
DM, % as-fed	92.45	93.67	95.02	95.23	94.87	97.58	98.00	96.82	95.73	94.64	93.54	92.45
OM, % DM	87.92	86.05	91.63	86.49	84.18	95.35	85.07	88.20	88.13	88.06	87.99	87.92
WSC, % DM	13.40	17.63	9.80	21.92	21.86	6.21	18.32	15.46	14.95	14.43	13.91	13.40
NDF, % DM	37.75	28.94	43.43	29.69	20.13	49.11	21.63	30.29	32.16	34.02	35.89	37.75
ADF, % DM	21.91	21.98	19.91	38.27	23.82	38.27	20.95	27.68	25.51	25.51	23.16	21.91
CP, % DM	23.55	20.68	20.28	19.64	17.81	17.01	15.74	16.85	18.53	20.20	21.88	23.55

<sup>1</sup> Experiment 1 substrates as comprised on a DM basis as follows: 0; control substrate of 0% other substrate and 100% ryegrass, 50 Chicory; substrate of 50% chicory and 50% ryegrass, 50 Alfalfa; substrate comprised of 50% alfalfa and 50% ryegrass, 50 Plantain; substrate of 50% plantain and 50% ryegrass, 100 Chicory; 100% chicory substrate, 100 Alfalfa; 100% alfalfa substrate, 100 Plantain; 100% plantain substrate.

Experiment 2 substrates as comprised on a DM basis as follows: Ryegrass 0; substrate of 33% chicory, 33% alfalfa, and 33% plantain, Ryegrass 25; substrate of 25% ryegrass, 25% chicory, 25% alfalfa, and 25% plantain, Ryegrass 50; substrate of 50% ryegrass, 16% chicory, 16% alfalfa, and 16% plantain, Ryegrass 75; substrate of 75% ryegrass, 8 % chicory, 8% alfalfa, and 8% plantain, Ryegrass 100; control substrate of 100% ryegrass.

**Table 3.2** *In vitro* gas production and fermentation outputs for experiment 1: different inclusion levels (0, 50, and 100%) of chicory, plantain, or alfalfa, with ryegrass after 24 hr incubation within ANKOM<sup>RF</sup> Gas Production Systems.

Item <sup>2</sup>	Treatments <sup>1</sup>								Linear Contrast			Quadratic Contrast		
	0	50			100			SEM <sup>3</sup>	Chicory	Alfalfa	Plantain	Chicory	Alfalfa	Plantain
		Chicory	Alfalfa	Plantain	Chicory	Alfalfa	Plantain							
pH	6.35	6.33 <sup>a</sup>	6.36 <sup>a</sup>	6.21 <sup>b</sup>	6.24 <sup>b</sup>	6.37 <sup>a</sup>	6.05 <sup>c</sup>	0.04	<0.01	0.61	<0.01	0.11	0.84	0.70
App. DMD	72.66	74.15 <sup>a</sup>	60.87 <sup>b</sup>	69.33 <sup>ab</sup>	84.53 <sup>a</sup>	49.11 <sup>b</sup>	78.26 <sup>a</sup>	6.00	<0.01	<0.01	0.17	0.24	0.81	0.11
NH <sub>3</sub> , mmol/L	22.84	20.84 <sup>ab</sup>	21.70 <sup>a</sup>	16.83 <sup>b</sup>	16.28 <sup>a</sup>	20.54 <sup>a</sup>	10.16 <sup>c</sup>	1.63	<0.01	0.21	<0.01	0.47	0.99	0.84
24 hr gas, ml/ gDM	90.75	102.45	91.92	93.62	116.50 <sup>a</sup>	95.63 <sup>b</sup>	107.47 <sup>ab</sup>	7.42	<0.01	0.40	<0.01	0.83	0.81	0.30
24 hr gas, ml/ g OM	101.96	119.07	101.25	108.23	138.68 <sup>b</sup>	100.30 <sup>a</sup>	126.34 <sup>ab</sup>	8.36	<0.01	0.40	<0.01	0.83	0.81	0.30
Total VFA, mmol/L	62.51	66.33	64.54	63.66	78.79 <sup>a</sup>	63.38 <sup>b</sup>	64.34 <sup>b</sup>	2.95	<0.01	0.86	0.71	0.34	0.72	0.96
VFA Conc., mmol/L														
Acetate	40.17	43.34	43.09	41.98	51.51	42.70	43.37	6.06	<0.01	0.49	0.39	0.46	0.63	0.95
Propionate	13.12	13.91	12.49	13.32	17.28 <sup>a</sup>	11.41 <sup>b</sup>	12.74 <sup>b</sup>	1.32	<0.01	0.14	0.74	0.22	0.83	0.71
Butyrate	6.03	6.26	5.80	5.93	7.30 <sup>a</sup>	5.94 <sup>b</sup>	6.27 <sup>ab</sup>	0.94	0.02	0.86	0.64	0.39	0.70	0.63
Valerate	1.06	0.97	1.02	0.84	0.98 <sup>a</sup>	1.03 <sup>a</sup>	0.70 <sup>b</sup>	0.23	0.37	0.70	<0.01	0.53	0.73	0.60
Iso-butyrate	0.70	0.62	0.71	0.52	0.56 <sup>ab</sup>	0.69 <sup>a</sup>	0.40 <sup>b</sup>	0.09	0.10	0.85	<0.01	0.84	0.86	0.66
Iso-valerate	1.17	0.99	1.18	0.82	0.88 <sup>b</sup>	1.28 <sup>a</sup>	0.59 <sup>b</sup>	0.21	0.10	0.53	<0.01	0.83	0.78	0.71
Hexanoate	0.25	0.25	0.24	0.26	0.28	0.33	0.27	0.15	0.64	0.23	0.81	0.83	0.46	0.95
VFA Prop.														
Acetate	63.77	65.32	66.51	65.96	65.19	67.09	67.38	1.53	0.32	0.02	0.01	0.53	0.42	0.78
Propionate	21.58	21.10	19.79	21.00	22.25	18.74	20.12	1.97	0.67	0.07	0.35	0.57	0.79	0.92
Butyrate	9.67	9.37	8.92	9.27	9.24	9.14	9.62	0.39	0.27	0.18	0.90	0.82	0.18	0.29
Valerate	1.67	1.44 <sup>a</sup>	1.54 <sup>a</sup>	1.28 <sup>b</sup>	1.20 <sup>b</sup>	1.54 <sup>a</sup>	1.04 <sup>c</sup>	0.16	<0.01	0.02	<0.01	0.98	0.16	0.14
Iso-butyrate	1.12	0.93 <sup>b</sup>	1.11 <sup>a</sup>	0.82 <sup>b</sup>	0.70 <sup>b</sup>	1.09 <sup>a</sup>	0.61 <sup>b</sup>	0.06	<0.01	0.64	<0.01	0.80	0.97	0.48
Iso-valerate	1.83	1.49	1.82	1.29	1.09 <sup>b</sup>	1.97 <sup>a</sup>	0.86 <sup>b</sup>	0.13	<0.01	0.42	<0.01	0.86	0.56	0.68
Hexanoate	0.34	0.36	0.33	0.38	0.32	0.43	0.36	0.19	0.82	0.23	0.76	0.72	0.39	0.67

<sup>1</sup> Substrates comprised on a DM basis as follows: 0; control substrate of 100% ryegrass, 50 Chicory; substrate of 50% chicory and 50% ryegrass, 50 Alfalfa; substrate comprised of 50% alfalfa and 50% ryegrass, 50 Plantain; substrate of 50% plantain and 50% ryegrass, 100 Chicory; 100% chicory substrate, 100 Alfalfa; 100% alfalfa substrate, 100 Plantain; 100% plantain substrate.

<sup>2</sup> App. DMD; apparent dry matter digestibility, NH<sub>3</sub>; rumen ammonia, Total VFA; total volatile fatty acid concentration, VFA Conc.; volatile fatty acid concentration, VFA Prop.; volatile fatty acid proportion.

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

**Table 3.3** P-values from ANOVA for treatment (chicory, plantain, or alfalfa), level of inclusion (0, 50, and 100%), and treatment × level of inclusion for the *in vitro* fermentation outputs for experiment 1 and treatment (0, 25, 50, 75, and 100% inclusion of mixture) for experiment 2 after 24 hr incubation within ANKOM<sup>RF</sup> Gas Production Systems.

Item <sup>1</sup>	Experiment 1			Experiment 2
	Treatment	Level	Treatment × Level	Treatment
pH	<0.01	<0.01	<0.01	<0.01
App. DMD	<0.01	0.14	<0.01	0.14
NH <sub>3</sub> , mmol/L	<0.01	<0.01	<0.01	<0.01
24 hr gas, ml/ gDM	0.04	<0.01	0.14	<0.01
24 hr gas, ml/ g OM	<0.01	<0.01	<0.01	<0.01
Total VFA, mmol/L	0.14	0.08	0.14	0.48
VFA Conc., mmol/L				
Acetate	0.35	0.03	0.39	0.43
Propionate	<0.01	0.55	<0.01	0.43
Butyrate	0.19	0.19	0.42	<0.01
Valerate	0.02	<0.01	0.13	0.13
Iso-butyrate	0.03	<0.01	0.20	<0.01
Iso-valerate	0.01	0.03	0.09	<0.01
Hexanoate	0.93	0.48	0.94	0.62
VFA Prop.				
Acetate	0.49	<0.01	0.82	0.05
Propionate	0.31	0.33	0.60	0.24
Butyrate	0.55	0.07	0.91	<0.01
Valerate	<0.01	<0.01	<0.01	<0.01
Iso-butyrate	<0.01	<0.01	<0.01	<0.01
Iso-valerate	<0.01	<0.01	<0.01	<0.01
Hexanoate	0.86	0.75	0.75	<0.01

<sup>1</sup>App. DMD; apparent dry matter digestibility, NH<sub>3</sub>; rumen ammonia, Total VFA; total volatile fatty acid concentration of the rumen, VFA Conc.; volatile fatty acid concentration, VFA Prop.; volatile fatty acid proportion.

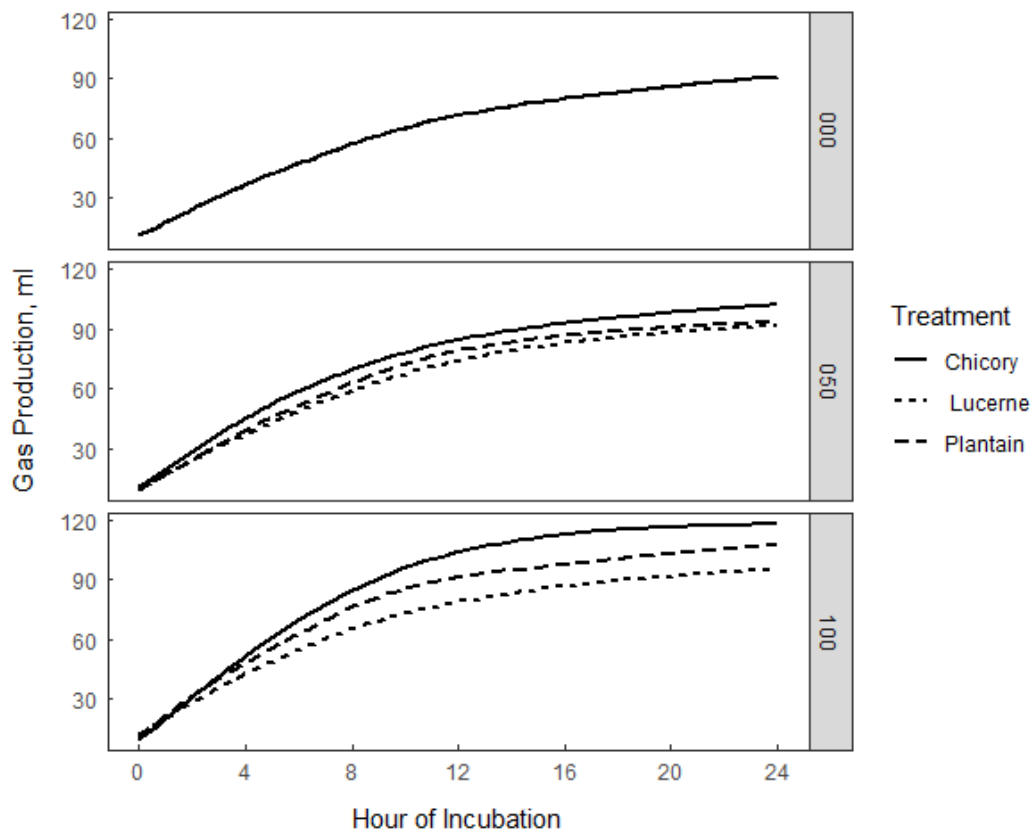
**Table 3.4** In vitro gas production and fermentation output information for experiment 2 on the effect of different proportions of ryegrass with chicory, plantain, and alfalfa after 24h incubation within ANKOM<sup>RF</sup> Gas Production Systems.

Item <sup>2</sup>	Treatments <sup>1</sup>					SEM	Linear Contrast		
	RG100	RG75	RG50	RG25	RG0		Linear	Quadratic	Cubic
pH	6.30	6.27	6.24	6.17	6.19	0.04	<0.01	0.59	0.14
App. DMD	67.73	64.12	62.47	80.94	73.20	7.19	0.13	0.58	0.13
NH <sub>3</sub> , mmol/L	24.60	20.86	20.65	17.28	15.79	0.70	<0.01	0.61	0.46
24 hr gas production, ml/ g DM	97.91 <sup>b</sup>	102.29 <sup>ab</sup>	103.77 <sup>ab</sup>	105.96 <sup>a</sup>	108.60 <sup>a</sup>	7.53	0.22	0.20	<0.01
24 hr gas, ml/ g OM	111.36 <sup>b</sup>	116.26 <sup>ab</sup>	117.84 <sup>ab</sup>	120.23 <sup>a</sup>	123.13 <sup>a</sup>	8.56	0.24	0.21	<0.01
Total VFA, mmol/L	73.87	76.18	74.90	80.09	79.30	6.15	0.12	0.98	0.79
VFA Conc., mmol/L									
Acetate	50.46	52.35	51.72	55.09	54.54	4.17	0.09	0.91	0.83
Propionate	13.02	13.33	12.92	13.98	14.01	1.13	0.12	0.65	0.86
Butyrate	6.54 <sup>b</sup>	6.80 <sup>ab</sup>	6.92 <sup>ab</sup>	7.71 <sup>a</sup>	7.63 <sup>a</sup>	0.73	<0.01	0.98	0.40
Valerate	1.16	1.10	1.02	1.04	1.01	0.11	0.02	0.33	0.84
Iso-butyrate	0.90 <sup>a</sup>	0.86 <sup>ab</sup>	0.78 <sup>ab</sup>	0.77 <sup>ab</sup>	0.70 <sup>b</sup>	0.04	<0.01	0.89	0.94
Iso-valerate	1.43 <sup>a</sup>	1.39 <sup>a</sup>	1.22 <sup>ab</sup>	1.18 <sup>ab</sup>	1.06 <sup>b</sup>	0.07	<0.01	0.95	0.77
Hexanoate	0.37	0.35	0.33	0.34	0.34	0.08	0.30	0.24	0.96
VFA Prop.									
Acetate	68.38 <sup>b</sup>	68.70 <sup>ab</sup>	69.10 <sup>a</sup>	68.78 <sup>ab</sup>	68.76 <sup>ab</sup>	0.48	0.10	0.02	0.67
Propionate	17.58	17.52	17.21	17.47	17.69	0.33	0.72	0.05	0.65
Butyrate	8.80 <sup>d</sup>	8.92 <sup>c</sup>	9.20 <sup>b</sup>	9.59 <sup>a</sup>	9.59 <sup>a</sup>	0.28	<0.01	0.47	<0.01
Valerate	1.57 <sup>a</sup>	1.44 <sup>b</sup>	1.36 <sup>c</sup>	1.29 <sup>d</sup>	1.27 <sup>d</sup>	0.05	<0.01	<0.01	0.92
Iso-butyrate	1.23 <sup>a</sup>	1.14 <sup>b</sup>	1.05 <sup>c</sup>	0.97 <sup>d</sup>	0.90 <sup>e</sup>	0.10	<0.01	0.49	0.86
Iso-valerate	1.95 <sup>a</sup>	1.83 <sup>a</sup>	1.65 <sup>b</sup>	1.49 <sup>c</sup>	1.37 <sup>c</sup>	0.18	<0.01	0.94	0.30
Hexanoate	0.48 <sup>a</sup>	0.45 <sup>ab</sup>	0.43 <sup>b</sup>	0.41 <sup>b</sup>	0.42	0.07	<0.01	0.02	0.54

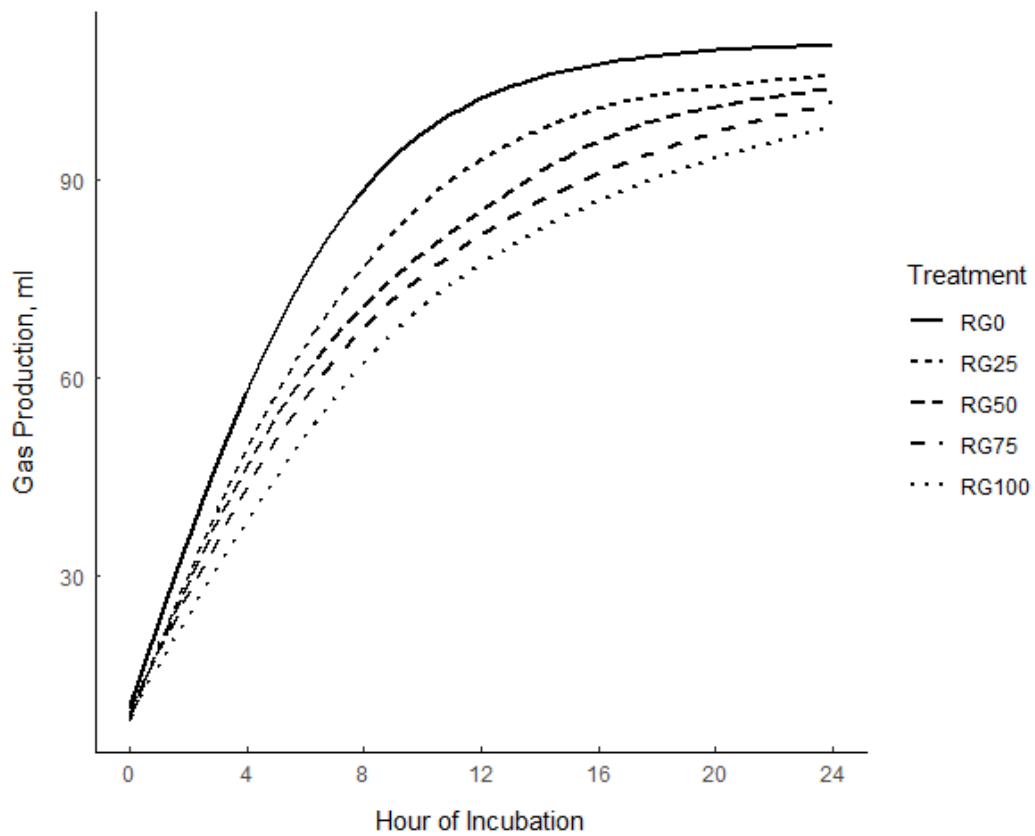
<sup>1</sup>Experiment 2 substrates as comprised on a DM basis as follows: Ryegrass 0; substrate of 33% chicory, 33% alfalfa, and 33% plantain, Ryegrass 25; substrate of 25% ryegrass, 25% chicory, 25% alfalfa, and 25% plantain, Ryegrass 50; substrate of 50% ryegrass, 16% chicory, 16% alfalfa, and 16% plantain, Ryegrass 75; substrate of 75% ryegrass, 8 % chicory, 8% alfalfa, and 8% plantain, Ryegrass 100; control substrate of 100% ryegrass.

<sup>2</sup>App. DMD; apparent dry matter digestibility, NH<sub>3</sub>; rumen ammonia, Total VFA; total volatile fatty acid concentration of the rumen, VFA Conc.; volatile fatty acid concentration, VFA Prop.; volatile fatty acid proportion.

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

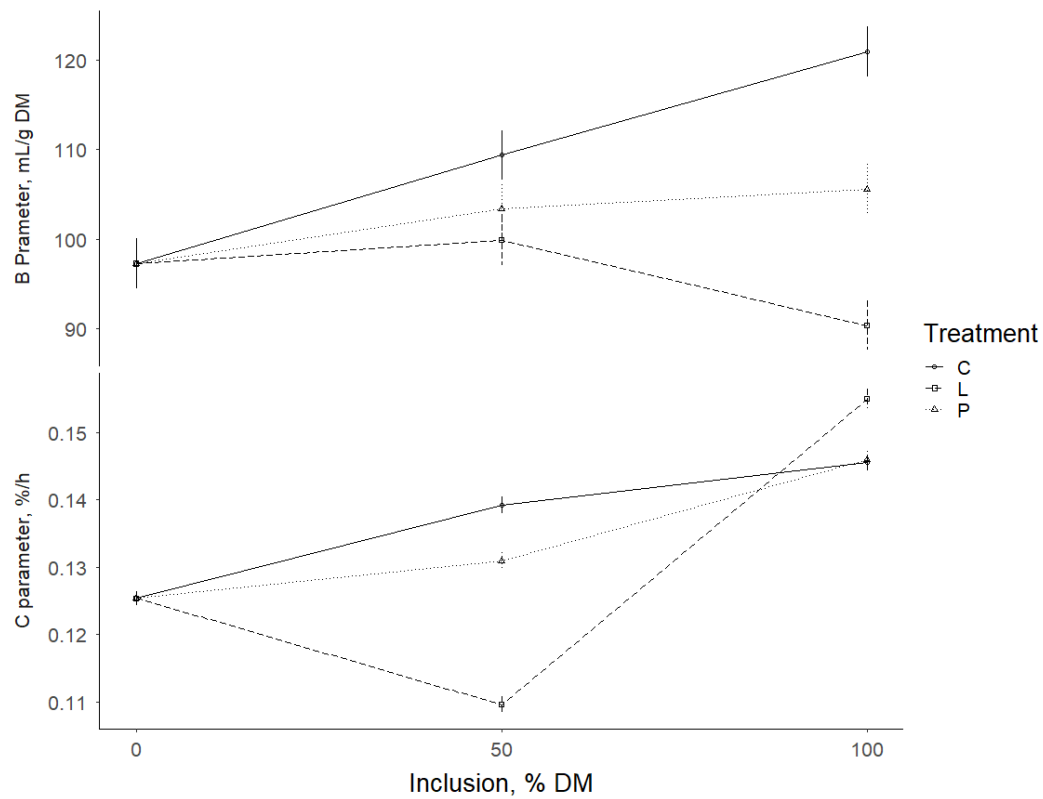


**Figure 3.1** *In vitro* gas production from at different levels of inclusion of chicory, plantain, or plantain alfalfa (0, 50, and 100%) with ryegrass. Note: as these values were the product of fermenting 1 g of DM (corrected for residual DM) the results could also be interpreted as ml/ g DM.

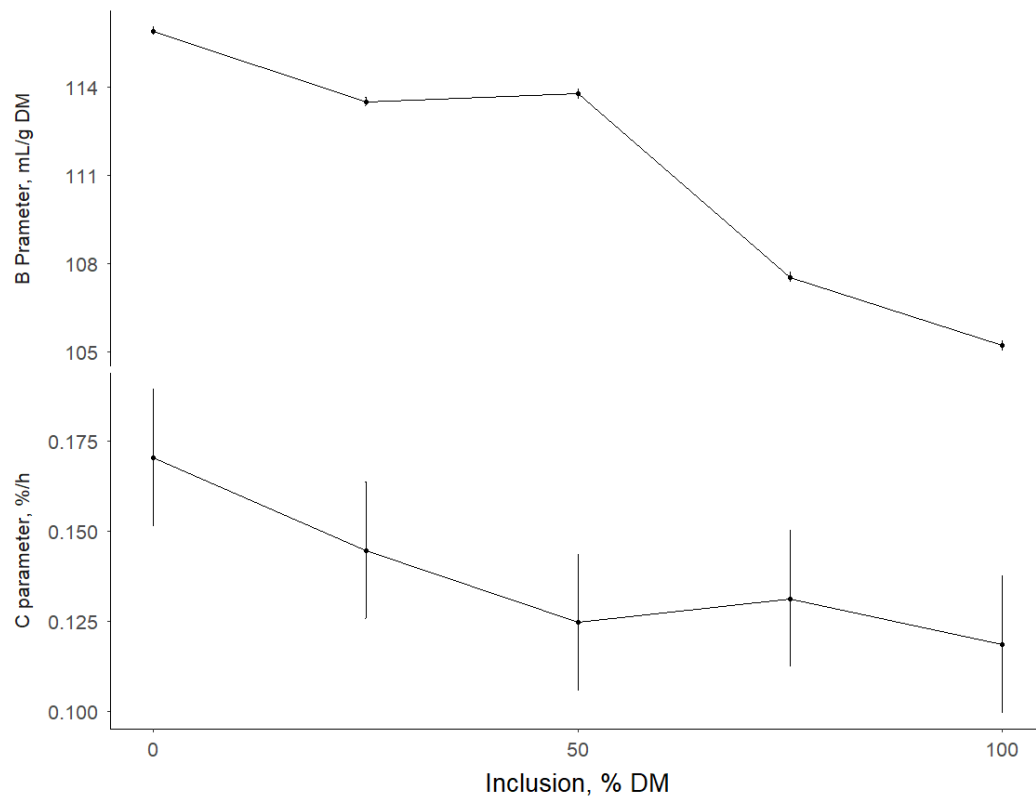


**Figure 3.2** *In vitro* gas production from treatment substrate comprised of different proportions of ryegrass with the remaining substrate comprised of equal parts chicory, plantain, and alfalfa. Note: as these values were the product of fermenting 1 g of DM (corrected for residual DM) the results could also be interpreted as ml/ g DM.

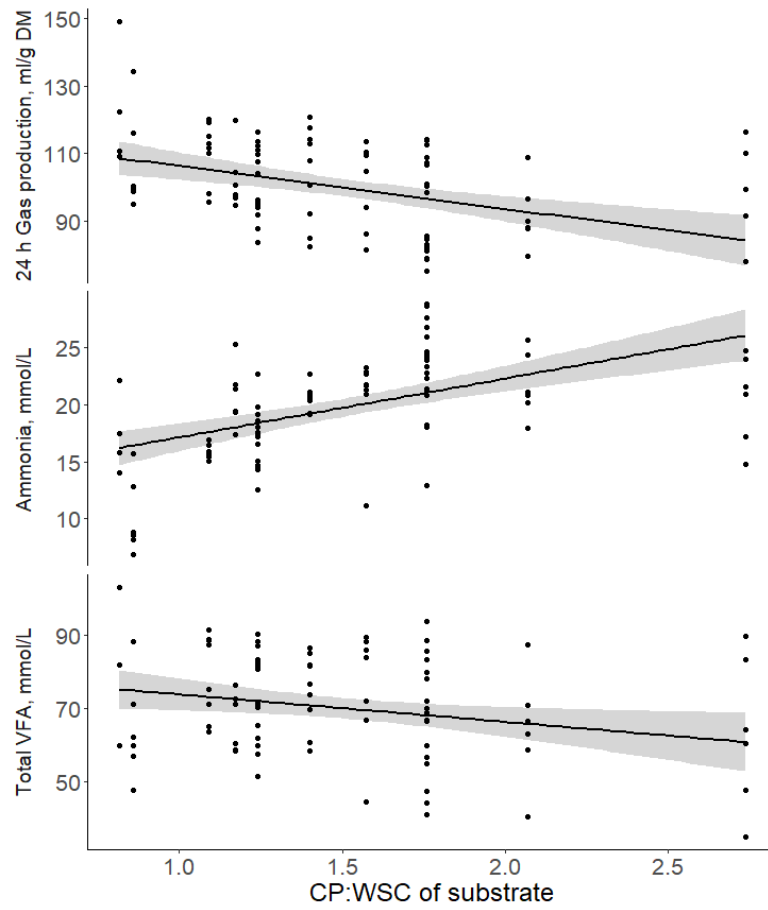




**Figure 3.3** Gas production constants from the Ørskov non-linear equation:  $p = b(1 - e^{-ct})$  of ryegrass with increasing inclusion of chicory, alfalfa, or plantain. In which  $b$  is the theoretical asymptote of the gas curve,  $c$  is the fractional rate of gas production (%/hr),  $p$  is the gas production after time 't'. Inclusion; the level of inclusion of each 'Treatment' species with the remaining dry matter being comprised of ryegrass. Treatment: C Chicory, L Alfalfa, P Plantain.



**Figure 3.4** Gas production constants from the nonlinear equation:  $p = b(1 - e^{-ct})$  of ryegrass with different levels of inclusion of ryegrass with remaining substrate comprised of equal parts chicory, plantain, and alfalfa. In which  $b$  is the theoretical asymptote of the gas curve,  $c$  is the fractional rate of gas production (%/hr),  $p$  is the gas production after time 't'.



**Figure 3.5** Total volatile fatty acid (VFA), ammonia, and gas production after 24 hr fermentation as the crude protein (CP): water soluble carbohydrate (WSC) ratio of substrate fermenting increases.

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## Chapter 4

# Functional diversity vs. monotony: the effect of a multiforage diet as opposed to a single forage diet on animal intake, performance, welfare, and urinary nitrogen excretion

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### 4.1 Abstract

I determined the effect of offering animals a multiforage choice (MF) of fresh herbages on dry matter intake (DMI), live weight gain, and animal welfare in comparison with a monotonous diet of ryegrass (*Lolium perenne L.*). Twenty ram lambs ( $30.5 \pm 0.9$  kg initial live weight; mean  $\pm$  SEM) were randomly allocated to either a diet consisting of diverse MF choice or a single forage ryegrass (SF) diet ( $n = 10$  per treatment) for 35 d. Lambs were penned individually indoors and herbage was harvested and fed to animals in fed bins within their pens. Both diets were fed ad libitum; however, the MF diet was composed of set dry matter ratios of 24% chicory (*Cichorium intybus L.*), 30% alfalfa (*Medicago sativa L.*), 25% plantain (*Plantago lanceolata L.*), and 21% ryegrass. The DMI of the MF lambs was 48% greater ( $P < 0.01$ ) and the within animal day-to-day coefficient of variation (CV) of intake was 26% lower ( $P < 0.01$ ) than the SF lambs. The average daily gain (ADG) of lambs offered the MF diet was 92% greater ( $P < 0.01$ ) than the lambs offered the SF diet. The within-animal day-to-day CV of intake was negatively related to ADG ( $r = -0.59$ ;  $P < 0.01$ ). The MF lamb's urinary N concentration was 30% lower ( $P < 0.01$ ) than that of the SF lambs. The SF lambs spent more time ( $P < 0.05$ ) exhibiting stereotypic behaviors in the afternoon and spent more time observing other animals than the MF. Overall, allocating an MF choice of fresh herbages as opposed to a single forage diet of ryegrass

increases DMI and thereby animal performance and welfare, while potentially reducing urinary N excretion.

**Key words:** diet, diversity, monotony, welfare, sheep

## 4.2 Introduction

The ancestors of today's ruminants evolved within environments containing a diverse array of plant species (Provenza et al., 2007). To compose their own diet within such diverse environments, individual animals selected from a multitude of unique forage species, of which availability, abundance, and chemical composition varied across space and through time (Provenza et al., 2007). This is in stark contrast with monotonous diets typically used by today's intensive pastoral livestock production systems, which generally offer binary mixes of a grass and a legume [e.g., perennial ryegrass (*Lolium perenne* L.) and white clover (*Trifolium repens* L.)]. Undoubtedly, such dietary strategies have helped increase productivity and profitability of pastoral production systems by simplifying agronomic and grazing management of swards. Mixes of a perennial ryegrass and a legume ("simple pastures") are widely fed to livestock, as perennial ryegrass is not only a palatable and digestible forage (Delagarde et al., 2000), but also provides high herbage yields under a range of temperate environments and management conditions (Moser et al., 1996). Simple pastures can result in a 'monotonous' diet, as over time biotic (e.g., competition) and abiotic (e.g., soil moisture) factors affect plant persistence as animals graze selectively so that only one species persists (Zydenbos et al., 2011; Gregorini et al., 2017). Moreover, the repeated allocation of such a single species pastures or simple pasture mixes induces dietary monotony, which is defined as tedious repetition or a lack of variety (Pearsall, 2001). Despite the advantages of 'simple pastures' in terms of grazing management, there has been little consideration of the consequences of monotony for animals.

Animal production, health, and, in turn, welfare can be compromised by dietary monotony, for example by inducing nutrient imbalances (Provenza et al., 2007; Hogan and Phillips, 2008). Nutrient imbalances can lead to phenomenon such as incidental restriction, where the upper threshold for a nutrient is reached and animals cease eating (i.e., nutrient-specific satiety), with an ensuing deficiency in nutrients present in lower concentrations within feeds (Raubenheimer, 1992; Provenza, 1995; Provenza, 1996; Gregorini et al., 2017). Conversely, incidental augmentation is encountered when animals consume nutrients present in higher concentrations to excessive and potentially detrimental quantities to satisfy other nutrient or energetic needs (Raubenheimer, 1992; Provenza, 1995; Gregorini et al., 2017). Impaired nutrition can have a number of detrimental effects on animal health and wellbeing (Beck and Gregorini, 2020). Furthermore, nutritionally imbalanced diets are contributors to inefficient utilization and excretion of nutrients to the environment,

therefore contributing to negative environmental footprint of pastoral production systems (Gregorini et al., 2017). Thereby, monotonous diets may have detrimental impacts on the environment as well as animal performance and welfare.

Providing ruminant livestock with diets diverse in flavor or biochemical composition increases dry matter intake (DMI) or feed conversion efficiency (FCE; Champion et al., 1998; Rogosic et al., 2006; Distel et al., 2007; Villalba et al., 2011), improves animal health (Provenza et al., 2007; Dixon and Pasinetti, 2010), and productivity (Rodríguez et al., 2007; Al-Marashdeh et al., 2020). Offering animal's choice from taxonomically diverse diets affords animals the opportunity to select plant combinations that meet their nutrition and therapeutic needs, while negating nutrients that are in excess or that are causing malaise (Villalba et al., 2010). Furthermore, providing choice from taxonomically diverse plants may provide greater benefits than when individual plant species are consumed alone (Tilman, 1982; Gregorini et al., 2017). Although the promising effects of dietary diversity on improved animal performance and productivity have been identified, much of this research has been conducted using concentrates or conserved forage, with little information regarding the effect of fresh forages. Therefore, we hypothesized that as opposed to a monotonous diet of perennial ryegrass, a taxonomically diverse MF diet would increase DMI, improve animal performance, reduce urinary N excretion, and enhance welfare. The objective of this study was to determine whether feeding animals a diet of equal proportions of cut fresh herbage: ryegrass, alfalfa (*Medicago sativa* L.), chicory (*Cichorium intybus* L.), and plantain (*Plantago lanceolata* L.) alter DMI, animal performance, N excretion, and animal welfare relative to a conventional monotonous diet of ryegrass.

### **4.3 Materials and Methods**

The study was conducted at the Johnstone Memorial Laboratory at Lincoln University (43°38'57"S, 172°27'01"E), according to the methods approved by the Lincoln University Animal Ethics Committee (AEC 2018–49) prior to experiment initiation.

#### **4.3.1 Animal Management and Dietary Treatments**

Twenty 6-month-old Coopworth rams [ $30.5 \pm 0.9$  kg initial live weight (LW); mean  $\pm$  SEM] were housed in individual pens (3.6  $\times$  1.0 m) indoors for 35 d starting on 4 March 2019. Animals were randomly allocated to one of two treatments (n = 10): single forage ryegrass (SF) or a multi-forage diet (MF) consisting of a selection of equal parts dry matter (DM) of ryegrass, alfalfa (*M. sativa* L.), chicory (*C. intybus* L.), and plantain (*P. lanceolata* L.). Ryegrass, chicory, and alfalfa were all in a vegetative state, whereas plantain contained 35% reproductive stem and seed head. The chicory was first year with a standing height of 35 cm prior to harvesting, ryegrass was at the three-leaf stage of

growth, and alfalfa ranged from mid to late vegetative state over the course of the trial (Hall, 1996). Animals offered the MF diet were presented all four feeds simultaneously, with each feed occupying one half of a split bin placed at each end of the pen. The half-bin that each forage species was offered in was randomly assigned for each pen. Both the SF and MF treatments received ad libitum access to fresh cut herbage of their respective diets at 0730 h and, if required, additional herbage was supplied at 1600 h. Herbage was cut using a Haldrup forage harvester (Haldrup GmbH, Ilshofen, Germany) that cut 3 cm above ground level. All herbages were stored at 4 °C until they were fed, any feed excess to daily requirement was stored for up to 2 d after harvest as a reserve. Herbage refusals from the previous day were weighed before each morning feeding. Each pen was cleaned daily, and water was freely available to animals at all times.

### **4.3.2 Animal Sampling and Measurements**

Samples of allocated herbage andorts were taken at each morning feeding time. All animals were weighed (Prattley 3-Way Manual Weigh Crate, Temuka, New Zealand with a Tru-test XR300 weigh head, Auckland, New Zealand) once weekly before morning feeding. Average daily gain (ADG) for each animal was determined as the slope of a regression line fitted for live weight across time for each individual animal. Samples of feces, blood, and rumen fluid were collected on days 1, 20, and 35. Fecal samples were collected by rectal grab. Blood samples were obtained by jugular venepuncture and collected with a 20G by 1" multisample collection needle (Greiner Bio-One, Kremsmünster, Austria) into a 10-mL heparinized blood tube (Greiner Bio-One, Kremsmünster, Austria). A 2-mL heparinized whole blood subsample was removed from the blood tube and stored at -20 °C until analysis. The remaining blood was centrifuged (Megafuge 1.0R, Heraeus Holding GmbH, Hanau, Germany) at 2,300 × g and 4 °C for 15 min; plasma was then aspirated and stored at -20 °C until analysis. Ruminal fluid was obtained via esophageal tubing. Ruminal fluid was subsampled into three containers: one acidified with sulphuric acid (10 µL of 98% sulphuric acid; Fisher Scientific, Loughborough, UK) and two without.

On days 23 and 24, five animals from each treatment were housed in metabolism crates for 48 hr, with the remaining animals housed within the crates on days 25 to 26, to determine total daily fecal and urine output. Representative samples of feces and urine were collected from the metabolism crates for each 24-hr period that animals were in the crates. Collection trays for urine contained ~250 mL of 5% sulphuric acid, so that the urine was immediately acidified to prevent ammonia (NH<sub>3</sub>) volatilization.

Behavioral observations were determined by scan sampling each animal at 2-min intervals and recording the displayed behavior, during daylight hours (0712 to 2010 h) on days 11 and 32 (Altmann, 1974; Villalba et al., 2015). During this daylight period, artificial lighting was used.

Behaviors recorded were: idle, eating, ruminating, pacing, chewing pen fixtures, head butting pen fixtures, head hanging, crouching, pawing or stamping, rearing, scratching, rubbing, observing other sheep, and observing humans (Table 4.1). These activities were of interest based on previous studies (Done-Currie et al., 1984; Lauber et al., 2012; Catanese et al., 2013). Pacing, chewing, head butting, head hanging, pawing or stamping, rearing, and crouching were grouped as stereotypic behaviors. Stereotypic behaviors are those that are repeated with no apparent function and are indicative of poor animal welfare (Broom, 1991; Catanese et al., 2013). Grooming was considered as the incorporation of scratching one's self and rubbing on pen fixtures as defined by Mattiello et al. (2019).

### 4.3.3 Sample Analysis

Herbage samples were frozen (-20 °C), freeze dried, ground by a centrifugal mill (ZM200; Retsch, Haan, Germany; 1mm screen), and analyzed using near infrared spectrophotometry (NIRS; Model: FOSS NIRS Systems 5000, MD) to determine chemical composition. Chemical composition values used for NIRS calibration were derived prior to sample analysis for DM (AOAC, 1990; method 930.15), organic matter (OM; 100%-ash%; AOAC, 1990; method 942.05), neutral detergent fiber (NDF; Van Soest et al., 1991), acid detergent fiber (ADF; AOAC, 1990; method 973.18), water-soluble carbohydrates (WSC; MAFF, 1986), digestible OM in DM (DOMD), DM digestibility (DMD), OM digestibility (OMD; lowerth et al., 1975), and crude protein (CP) by combustion (Variomax CN Analyser; Elementar Analysensysteme, Hanau, Germany). The NIRS calibration equations all had R<sup>2</sup> values greater than 0.97 and were within the calibration range. The metabolizable energy (ME) of herbage was estimated based on the Primary Industries Standing Committee (2007) equation:

$$[ME (MJ/kg DM) = \text{digestible OM in DM, \%}(DOMD) \times 0.16] \quad [1].$$

Fecal samples were frozen (-20 °C), freeze dried, ground to pass through a 1-mm screen using a centrifugal mill (ZM200; Retsch, Haan, Germany), and analyzed for total N by combustion (Variomax CN, Elementar Analysensysteme, Hanau, Germany). Due to a sampling error, fecal DM for individual animals was not available, and other research (Garrett et al., unpublished data) has shown no difference in fecal DM% between lambs provided similar dietary treatments (i.e., ryegrass compared with a diverse, herb containing diet). Thereby for the purpose of this work, an average fecal DM (20.23%) was assumed for calculating digestibility. Apparent dry matter digestibility (ADMD) was determined using the following equation:

$$ADMD = \frac{(DMI, kg/d - DM Faeces, kg/d)}{DMI, kg/d} \times 100 \quad [2].$$

Whole blood glutathione peroxidase (GPx), plasma total antioxidant status (TAS), and urine urea concentrations were measured with a Randox Rx Daytona clinical analyzer (Crumlin, Co. Antrim, UK) using kits RANSEL (Cat. No. RS504, Cat. No. NX2332, and Cat. No. UR3825, respectively). Urine total N was determined by combustion (Vario MAX CN, Elementar Analysensysteme, Hanau, Germany).

The NH<sub>3</sub> concentration of the acidified rumen samples was measured using the clinical analyzer (Randox Rx Daytona, Crumlin, Co. Antrim, UK) and a commercial test kit (Cat. No. AM3979; Randox; Crumlin, Co.) based on the enzymatic UV method described by Neeley and Phillipson (1988). The volatile fatty acid (VFA) concentration within non-acidified rumen samples was determined using a gas chromatograph (Shimadzu GC-2010, Kyoto, Japan with AOC-20i auto-sampler) fitted with a SGE BP21 30 m × 530 μm × 1 μm wide-bore capillary column as described by Chen and Lifschitz (1989). Rumen lactate concentration was analyzed by the Randox Rx Daytona clinical analyzer with a commercial kit (Cat. No. LC2389; Randox; Crumlin, Co.) using enzymatic determination of l-lactate

#### **4.3.4 Statistical analysis**

Statistical analyses were conducted using R (R Core Team, 2020, v.3.4.4). All data that were normally distributed ( $P > 0.10$ ; Shapiro–Wilk test) and had homogenous variance ( $P > 0.10$ ; Bartlett's test) were analyzed by analysis of variance (ANOVA) using the aov function of R. Data analyzed using aov function included: herbage chemical composition, DMI, ADG, FCE (ADG/ kg DMI), GPx, urinary urea, rumen, water consumption through feed, water intake, fecal water, urine water, and water out. If data was not normally distributed they were analyzed by a generalized linear model (GLM), using the glm function of R. Data analyzed using glm included: TAS, total nitrogen in urine, water drunk, water balance, and the proportion of water intake excreted as urine, feces, and accounted for by the water balance. Values for samples collected on day 1 were used as covariates for rumen, blood, fecal, and urine variables as these samples explained a significant ( $P < 0.05$ ) amount of variation. The ANOVA and GLM models that contained repeated measures (i.e., blood, urine, and fecal variables) included diet, day, and the diet × day interaction as fixed effects. The models for variables of averaged data or that were not repeatedly measured (i.e., rumen, DMI, ADG, and FCE) contained diet as fixed effects. Herbage chemical composition was assessed using a mixed model, using the lme function, with day as a random effect. Behavior data were averaged across observation days and analyzed by GLM using the glm function of R, with the distribution used for the model selected based on qq-plots of the residuals. The model for the animal behavior that was averaged across observation days included treatment, observation time (AM or PM), and their interaction as fixed effects. Upon significance of the ANOVA, means separation between diets were done using a pairwise t-test using the emmeans package (Lenth, 2018), when a multiple comparison was needed, such as when comparing more than two means which occurred in the event of a significant

interaction term for the repeated measures. Pearson's correlation coefficient between the day-to-day variability in DMI (CV) and DMI, ADG, and FCE was determined using the `cor.test` function of R. Statistical significance was declared at  $P \leq 0.05$  with tendencies declared at  $0.05 < P \leq 0.10$ .

## 4.4 Results

### 4.4.1 Diet Composition

The chemical composition of each herbage comprising the SF and MF diets is presented in Table 4.2. The CP content (24.9 %DM) of the ryegrass component of the SF and MF diet was 15%, 23%, and 39% greater than that of the chicory, alfalfa, and plantain, respectively ( $P < 0.05$ ). The WSC content of herbages decreased from chicory (22.0 %DM) to plantain and then ryegrass and alfalfa, which were not different to one another. These values were used to calculate the chemical composition of the total diet of each animal and the average chemical composition of each diet was compared between the dietary treatments (Table 4.3). There was no difference in ADF between the SF and MF diets ( $P > 0.10$ ). Ryegrass, which made up 100% the SF diet, had a greater ME ( $P = 0.04$ ) and greater DM, OM, OMD, NDF, and CP content compared with the average MF diet ( $P < 0.01$ ). However, the WSC content was greater for the MF diet compared with the SF diet ( $P < 0.01$ ).

### 4.4.2 Intake and Performance

The DMI of lambs fed MF was 48% greater ( $P < 0.01$ ) than lambs fed SF (Table 4.4). The DMI CV within animal between day was 26% greater ( $P < 0.01$ ) for the SF lambs than the MF lambs. In addition, the FCE (ADG/kg DMI) of lambs offered MF was 36% greater than the SF lambs ( $P < 0.01$ ). There was a negative correlation between the CV of day-to-day DMI and average DMI ( $r = -0.74$ ;  $P < 0.01$ ). The ADG of the MF lambs was 92% greater than that of the SF lambs (Table 4.4). Overall, there was a negative correlation between CV of day-to-day DMI and ADG ( $r = -0.60$ ;  $P < 0.01$ ) and no correlation ( $r = -0.37$ ;  $P = 0.11$ ) between CV of day-to-day DMI and FCE.

During the metabolism crate portion of this study, the DMI remained different between treatments ( $P < 0.01$ ; Table 4.5). However, the SF lambs showed a 0.06-kg decrease in DMI and the MF lambs consumed 0.14 kg more DM, compared with their average DMI over the trial. This resulted in a greater magnitude of difference between MF and SF for DMI, while in the metabolism crates, compared with when they were not (73% and 49% difference, respectively). The MF lambs tended to excrete 21% more feces than the SF lambs ( $P = 0.08$ ). Lambs offered MF had greater apparent DMD than SF lambs ( $P = 0.02$ ; Table 4.5).

### 4.4.3 Water and N Dynamics

Within the metabolism crates, SF lambs drunk 0.80 L/d of water, four times more than the MF (0.19 L/d; Table 4.5;  $P < 0.01$ ). However, the MF lambs consumed 80% more water from feed than the SF lambs ( $P < 0.01$ ). Total water consumption (water drunk + water from feed) of the MF lambs was 58% greater compared with the SF lambs ( $P < 0.01$ ). The MF lambs excreted 74% more urine than SF lambs ( $P < 0.01$ ). Water excreted within the feces did not differ between the treatments ( $P > 0.10$ ). The total amount of water excreted (64%) and the amount accounted for by the water balance (51%) was greater for the MF lambs compared with the SF lambs ( $P < 0.01$ ). Although the percentage of water intake excreted in the feces was greater for SF ( $10.29 \pm 1.40\%$ ) compared with MF ( $8.39 \pm 1.40\%$ ;  $P = 0.04$ ), there was no difference in how the percentage of total water intake was partitioned into urine ( $44.58 \pm 5.73\%$ ; mean  $\pm$  SEM) or the water balance ( $46.08 \pm 6.2\%$ ) between treatments ( $P > 0.10$ ).

The MF lambs consumed 51% more N than the SF lambs ( $P < 0.01$ ; Table 4.6). Apparent N digestibility tended to be greater for MF compared to SF ( $P = 0.09$ ). Although there was no difference in the amount (g/d) of urinary N excreted between treatments ( $P = 0.26$ ), the urine N concentration was 30% less for MF compared with the SF lambs ( $P < 0.01$ ). The MF lambs retained 15.38 g more N than the SF lambs ( $P < 0.01$ ). The percentage of the N consumed excreted in the feces was less for the MF ( $11.01 \pm 1.73\%$ ; mean  $\pm$  SEM) compared with SF ( $13.79 \pm 1.73\%$ ;  $P = 0.05$ ). Furthermore, MF ( $33.80 \pm 5.50\%$ ) tended to excrete a lower percentage of the consumed N in the urine compared with SF ( $42.60 \pm 5.50\%$ ;  $P = 0.08$ ). Consequently, the N retention was 12% greater for MF ( $55.19 \pm 6.97\%$ ) compared with SF ( $43.61 \pm 6.97\%$ ;  $P = 0.05$ ).

### 4.4.4 Rumen, Blood, and Plasma Parameters

There were no differences between treatments for rumen  $\text{NH}_3$  ( $P = 0.70$ ) or total VFA concentrations ( $P = 0.81$ ; Table 4.7). There was a tendency for SF lambs to have a greater acetate-to-propionate ratio compared with the MF lambs ( $P = 0.10$ ). The percentage of total VFA accounted for by valerate was greater for the MF lambs compared with the SF lambs ( $P = 0.02$ ). There were no differences between treatments in the percentage of VFA composed by propionate, iso-butyrate, butyrate, and iso-valerate ( $P > 0.10$ ). No differences were detected in the measured plasma and blood parameters. There was no difference in the levels of TAS for SF ( $1.11 \pm 0.03$  mmol/L; mean  $\pm$  SEM) and MF ( $1.16 \pm 0.03$  mmol/L;  $P = 0.17$ ) or in the GPx levels between the SF ( $36.7 \pm 589$  U/mL) and the MF ( $35.6 \pm 590$  U/mL;  $P = 0.21$ ).



#### **4.4.5 Behavioral Observations**

The proportion of time observed for each behavior is given in Table 4.8. For eating, there was a treatment × time interaction ( $P = 0.01$ ), whereby no difference was observed between treatments in the morning (0712 to 1200 h;  $P > 0.05$ ), but a 12% and 27% increase was observed in the afternoon compared with the morning for SF and MF, respectively ( $P < 0.01$ ). The MF lambs spent 17.1% more time eating in the afternoon than the SF lambs ( $P < 0.05$ ). For ruminating, there was an interaction between time and treatment whereby SF and MF lambs spent +2.7% and -6.5% time ruminating in the afternoon than the morning, respectively ( $P < 0.05$ ). Furthermore, in the afternoon, MF lambs spent 7.0% less time ruminating than SF lambs ( $P < 0.05$ ). For the percent of time spent idle, there was an effect of time and treatment ( $P = 0.01$ ), and a tendency for an interaction between the two terms ( $P = 0.09$ ). The SF lambs spent more time idle than MF lambs, regardless to the time of the day, and both treatments spent less time idle in the afternoon than the morning.

There was a treatment × time interaction on the percent of time spent displaying stereotypic behaviors ( $P = 0.05$ ). The SF lambs exhibited less stereotypic behaviors in the afternoon than in the morning ( $P < 0.05$ ), both of which were not different to the percentage of stereotypic behavior exhibited by the MF lambs in the morning ( $P > 0.10$ ). However, MF exhibited less stereotypic behaviors in the afternoon, compared with the morning and SF at any time of day ( $P < 0.05$ ). There were no differences for the percentage of time spent grooming ( $P > 0.10$ ). For the percent of time spent observing SF and MF, there was a treatment effect ( $P = 0.05$  and  $P < 0.01$ , respectively), with the MF lambs spending less time observing SF or MF lambs. For observing humans, there was an effect of time ( $P < 0.01$ ), reflecting an increased proportion of time observing humans in the morning compared with the afternoon.

### **4.5 Discussion**

#### **4.5.1 Animal ADG, DMI, and Digestibility**

Animal performance was enhanced by offering the MF diet compared with the SF diet. The ADG was 92% greater and FCE 36% greater for the MF lambs compared with the SF lambs, indicating that offering alternatives to a monotony of ryegrass can benefit animal production. These observations are consistent with previous reports in which lambs offered forages consisting of herbs and clover had a 200 g/d greater ADG than those on a sward composed of a single forage (Golding et al., 2011). In addition, Al-Marashdeh et al. (2020) reported that lambs grazing a three species sward had a 50% greater ADG than lambs grazing a ryegrass and white clover. However, results have been inconsistent. Raeside et al. (2017) fed spatially separated strips and different combinations of the same forage species used in the present study and reported no difference in ADG compared with

animals grazing a monoculture of alfalfa. This maybe a result of the 10-15% greater voluntary intake of legumes compared to grasses, as legumes have a lower resistance to chewing, faster digestion and thereby faster rumen throughput, reducing the effect of rumen fill (Fernandez-Turren et al., 2010). Further investigation is required to determine whether the effects reported here are ryegrass specific. Within the present study, the greater performance and FCE can be almost fully explained by the different levels of feed intake. Utilizing first principles based on energy requirements for maintenance and growth (Nicol and Brookes, 2007) and the measured diet quality, an estimated ADG of 102 and 224 g/d for the SF and MF treatments were predicted using the mean intakes of each treatment, values close to the actual gain measured. Furthermore, the greater FCE may reflect a dilution of maintenance requirement for the MF lambs due to their great intake. This is further supported by a modest (3%) difference in apparent DM digestibility between the diets and a lack of any effect of diet treatment on N digestibility or rumen NH<sub>3</sub> or VFA profiles, with the exception of valerate which only consisted of 1% of total VFA. As such, the benefit to animal performance appears to be due to greater levels of nutrient intake.

The reasons for the greater intake achieved by MF lambs are not fully apparent. The daily DM consumption was 3% and 4.5% of LW for the SF and MF, respectively, values for the latter being close to what may be expected as a physiological maximum. In part, this difference in intake may be explained by diet composition, apparent DMD, and NDF, which is associated with reductions in DMI due to rumen digesta outflow rates and thereby increasing the likelihood of physical limitation as an intake constraint (Mertens, 1994). Much of these differences in DMD are likely due to chemical and physical characteristics of the forages. For instance, the SF diet had 27.5% more NDF than the MF diet and dietary NDF have been reported to have a strong negative correlation with DMD (Du et al., 2016). The MF diet was ~50% herbs (i.e., plantain and chicory), which contain less structural cell arrangements, termed girder structures, than grasses. Thereby, differences in dietary DMD helped to facilitate the increment in DMI by lambs offered MF. However, the difference in apparent DMD between the two diets (89.6% and 85.4% for the MF and SF diets, respectively), was not of a magnitude that could fully explain the difference in DMI. In addition to expressing a greater DMI, the MF lambs exhibited a lower within animal day-to-day CV of DMI compared with the lambs provided the SF diet, indicating the animals offered the MF diet had a more consistent daily feed intake. Similar results were reported by Villalba et al. (2011), who found DMI was more consistent over time when given the choice of the same feed, but with different flavors, compared with a diet monotonous in flavor. Furthermore, our results present a strong negative correlation between CV and total intake, which is similar to the value of -0.82 derived from Ingvarsten et al. (1992). Our results showed that as CV was reduced, and DMI and ADG increased. Improved performance (i.e., ADG) with reduced CV has been reported by a number of studies (Allison, 1985; Galyean et al., 1992;

Horn et al., 2005; Williams et al., 2018), which allows the suggestion that the greater intake, and subsequent performance of MF was due to more consistent feed consumption. Although it remains unclear from the current study what the reasons for this may be, it may be speculated that a multitude of other factors including sensory and post-ingestive signals that also contribute to satiety and intake regulation, and likely contributed to the increased DMI of the MF lambs. It is possible that SF treatment resulted in nutrient-specific satiety or incidental restriction where intake ceased as one nutrient reached a physiological threshold despite other nutrient deficiencies existing (Raubenheimer, 1992; Early and Provenza, 1998; Gregorini et al., 2017). The SF may have resulted in satiety due to the repetitive oro-sensorial experience (i.e., taste and smell) as the intake related sensorial neurons response saturates and ceases for that particular feed or its nutrient profile, resulting in sensory specific satiety (Early and Provenza, 1998; Gregorini et al., 2017). Conversely, the joint intake of herbage comprising the MF lambs may have enabled greater intake by reducing the habituation of neurons (Epstein et al., 2009), through altering the consumption sequence. Such aversions occur even if the food closely matches animal needs as shown by Early and Provenza (1998), although aversions and within animal day-to-day CV of DMI become greater the less the food meets the needs of the animal. Therefore, due to the incomplete explanation of an increased DMI by MF lambs by dietary NDF and DMD, we argue that the treatment difference in DMI could be explained by a more integrated appreciation of the phytochemical diversity of the MF diet, that is, combination of basic nutritional and oro-sensorial factors.

#### **4.5.2 Nitrogen Dynamics**

Although the N intake was 51% greater for the MF compared with the SF lambs, there was no difference in the quantity of N excreted in the feces or urine; in fact, there was a tendency for a reduction in the proportion of consumed N excreted in the urine and a reduction of that excreted in the feces. A greater proportion of N consumed was retained (N use efficiency) by MF. Furthermore, urinary N concentration (g/L) was 30% lower for MF compared with SF, which suggests 30% reduction in N loading (kg N per ha) onto pasture at the urine patch level. Evidence for a difference in N loading at the patch level is strengthened by the same quantity of nitrogen excreted in a greater volume of urine over the day. There is a known curvilinear relationship between N loading at the urine patch level and N leaching (Di and Cameron, 2007; Li et al., 2012). This relationship suggests that the magnitude of difference between SF and MF could be 30%, or even greater, for nitrate leaching. Reductions in nitrogen deposition onto pastures is desirable as excess N has negative environmental impacts due to volatilization into the potent greenhouse gas nitrous oxide or by directly leaching into ground water, reducing water quality, and causing eutrophication (Cameron et al., 2013). Although much research is concentrating on reducing the environmental impact of cattle. Pasture based livestock production systems will be encouraged to reduce their environmental

impacts. Our results show that a dietary solution of multiple forages can be used to reach this goal while improving animal performance.

### **4.5.3 Welfare and Behavioral Observations**

There was some evidence that lambs offered the MF diet had improved welfare status. Animals in the MF treatment had greater ADG, which is a proxy for welfare status (Barrell, 2019) and there was a reduction in day-to-day CV of DMI, which has been linked to improvements in animal health and welfare (McGuffey et al., 1997, Forbes, 2007). For example, McGuffey et al. (1997) reported a 4% increase in the prevalence of adverse health incidents (e.g., metabolic or digestive disorder) in cows for every 1% increase in day-to-day intake CV within the first 21 d of lactation. However, there was no effect of treatment on the spot samples of TAS or GPx, and only modest effects on observed behaviors. The MF animals spent less time displaying stereotypic behaviors in the afternoon than the SF animals. Although some speculate that stereotypies help animals cope with their environment, the general consensus is that stereotypies are indicative of poor welfare (Broom, 1991). Although the proportion of the day spent conducting stereotypies is small, there would be more concern for animals spending a greater percentage of time exhibiting stereotypic behavior (Philbin, 1998). We believe such behaviors should be minimized where possible. We speculate that a potential cause for this behavioral difference may have been that the environment of the MF lambs was more stimulating, as they had choice from different flavors, textures, and “make your own” feed combinations, allowing more freedom to express individual personality and normal behaviors. Furthermore, time spent observing other sheep, which was greater for SF, has been suggested as a mechanism to alleviate boredom in an environment with fewer stimulations (Done-Currie et al., 1984). Conversely, less time observing sheep has also been suggested as withdrawn and non-alert state to alleviate the stress of a situation (Done-Currie et al., 1984). However, when we consider that MF spent more time conducting other “busy” activities (i.e., eating) and less time idle, and exhibited other signs of improved welfare (e.g., greater ADG), the former explanatory speculation is more likely. Again, although the proportion of the day accounted for by such observatory behaviors is small, statistically our results suggest a treatment difference between the MF and SF lambs for such indicators of welfare, indicating further research and refined measurement is needed. Our findings stress the importance of considering of both physiological and behavioral measurements when assessing animal welfare, to build the most complete picture possible.

## **4.6 Conclusions**

Providing animals with a multiforage diet that is taxonomically diverse can provide win-win-win situations by increasing DMI and reducing day-to-day variability of intake, improving

performance (ADG), and reducing the environmental impact by lowering urinary N excretion, while potentially improving animal welfare. The improvements to animal performance from a multiforage diet have some nutritional basis; however, further research is required on the potential mechanisms for improved intake and mild behavioral and welfare differences we detected. Our results provide the basis and outline of potential benefits to designing and establishing functionally diverse pastures; however, more research is required with different plant species in different grazing settings.

**Table 4.1** Ethogram of recorded behavioral activities.

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Behavior	Description
Eating	Eating (specific feed was recorded)
Idle	Sheep not engaged in any of the following behaviors
Ruminating	Sheep is ruminating
Pacing	Walking in a distinct pattern, such as frequent walking back and forth, weaving, or moving in circles
Chewing pen fixtures	Chewing pen fixtures (e.g. feed bin, bars)
Head butting pen fixtures	Butting pen fixtures
Head hanging	Standing quietly with head drooped down
Crouching	Crouching in fear (usually to human activity)
Pawing or stamping	Striking ground with forelegs
Rearing	Head raised with forelegs on pen or off ground, back legs on ground
Scratching	Scratching self
Rubbing	Rubbing on pen fixtures
Observing other sheep	In an alert state, ears pricked, or actively looking with attention directed to other sheep (The treatment of the animal being observed was recorded)
Observing humans	In an alert state, ears pricked, or actively looking with attention directed to other sheep

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**Table 4.2** Chemical composition of the herbage composing the single forage (perennial ryegrass only) and a taxonomically diverse multi-forage choice (MF) diet of equal proportions of fresh cut herbage of ryegrass, alfalfa (*Medicago sativa* L.), chicory (*Cichorium intybus* L.), and plantain (*Plantago lanceolata* L.).

Item <sup>1</sup>	Herbage				SEM <sup>2</sup>
	Ryegrass	Alfalfa	Plantain	Chicory	
ME, MJ/kg of DM	11.0 <sup>b</sup>	8.8 <sup>c</sup>	10.8 <sup>b</sup>	13.2 <sup>a</sup>	0.3
DM, % as-is	19.7 <sup>a</sup>	21.7 <sup>a</sup>	14.3 <sup>b</sup>	8.7 <sup>c</sup>	0.1
OM, % DM	90.6 <sup>b</sup>	93.8 <sup>a</sup>	88.5 <sup>c</sup>	86.4 <sup>d</sup>	0.8
OMD, % DM	76.9 <sup>b</sup>	58.9 <sup>c</sup>	74.5 <sup>b</sup>	91.9 <sup>a</sup>	2.1
WSC, % DM	7.9 <sup>c</sup>	5.7 <sup>c</sup>	13.1 <sup>b</sup>	22.0 <sup>a</sup>	1.9
NDF, % DM	49.5 <sup>a</sup>	46.0 <sup>a</sup>	30.3 <sup>b</sup>	16.1 <sup>c</sup>	2.2
ADF, % DM	26.1 <sup>b</sup>	35.9 <sup>a</sup>	24.5 <sup>b</sup>	17.5 <sup>c</sup>	1.2
CP, % DM	24.9 <sup>a</sup>	20.3 <sup>bc</sup>	17.9 <sup>c</sup>	21.7 <sup>b</sup>	1.3

<sup>a-c</sup> Means in a row with different superscripts are statistically different ( $P < 0.05$ ).

<sup>1</sup> ME, metabolizable energy; DM, dry matter; OM, organic matter; OMD, OM digestibility; WSC, water-soluble carbohydrates; NDF, neutral detergent fiber; ADF, acid detergent fiber; CP, crude protein.

<sup>2</sup> SEM, standard error of the mean.

**Table 4.3** Chemical composition of the monotonous diet (perennial ryegrass only, SF) and the calculated chemical composition of a taxonomically diverse multi-forage choice (MF) diet of equal proportions of fresh cut herbage of perennial ryegrass (*Lolium perenne* L.), alfalfa (*Medicago sativa* L.), chicory (*Cichorium intybus* L.), and plantain (*Plantago lanceolata* L.) offered to the ram lambs.

Item <sup>1</sup>	Treatment Diet		SEM <sup>2</sup>	P-value <sup>3</sup>
	SF	MF		
ME, MJ/kg DM	11.0	10.8	0.1	0.04
DM, % DM	19.7	18.5	0.2	<0.01
DMD <sub>p</sub> , % DM	73.3	71.5	0.6	<0.01
OM, % DM	90.6	90.1	0.2	<0.01
OMD, % DM	76.9	74.4	0.7	<0.01
WSC, % DM	7.9	11.8	0.3	<0.01
NDF, % DM	49.5	35.9	0.6	<0.01
ADF, % DM	26.1	26.7	0.4	0.17
CP, % DM	24.7	21.1	0.1	<0.01

<sup>1</sup> ME, Metabolisable energy; DM, Dry matter; DMD<sub>p</sub>, DM digestibility predicted by Near infrared spectroscopy; OM, organic matter; OMD, OM digestibility; WSC, water soluble carbohydrates; NDF, neutral detergent fiber; acid detergent fiber; CP, crude protein.

<sup>2</sup> SEM, standard error of the mean.

<sup>3</sup> t-test P-value.

Note: values for diverse diet chemical composition were calculated by using the percentage of the Item value that each dietary component accounted for.

**Table 4.4** Dry matter intake and growth performance of Coopworth lambs fed for a 35-d period either a monotonous diet (perennial ryegrass only, SF) or a taxonomically diverse MD diet of equal proportions of fresh cut herbage of perennial ryegrass (*Lolium perenne* L.), alfalfa (*Medicago sativa* L.), chicory (*Cichorium intybus* L.), and plantain (*Plantago lanceolata* L.).

Item <sup>1</sup>	Treatments		SEM <sup>2</sup>	P-value <sup>3</sup>
	SF	MF		
Initial LW, kg	31	30	0.9	0.46
DMI, kg/d	0.99	1.47	0.01	<0.01
DMI CV, %	21.3	15.8	0.8	<0.01
Daily gain, g LW/d	97	187	10	<0.01
FCE, g LWgain/ kg DMI	89	121	7	<0.01

<sup>1</sup> Initial LW, initial live weight; DMI, dry matter intake; DMI CV, day-to-day DMI co-efficient of variation; FCE, feed conversion efficiency.

<sup>2</sup> SEM, standard error of the mean.

<sup>3</sup> t-test P-value.



**Table 4.5** Dry matter intake, water consumption, and fecal and urine output information obtained from a 48-hr period within metabolism crates of Coopworth lambs offered a monotonous diet (perennial ryegrass only, SF) or a taxonomically diverse multi-forage choice (MF) diet of equal proportions of cut fresh herbage of perennial ryegrass (*Lolium perenne* L.), alfalfa (*Medicago sativa* L.), chicory (*Cichorium intybus* L.), and plantain (*Plantago lanceolata* L.)

Item <sup>1</sup>	Treatments		SEM <sup>2</sup>	P-value <sup>3</sup>
	SF	MF		
DMI, kg/d	0.93	1.61	0.06	<0.01
Feces, kg DM/d	0.14	0.17	0.01	0.07
ADMD, % DM	85.18	88.79	2.34	<0.01
Water intake, L/d				
Trough	0.80	0.19	0.23	<0.01
Feed	4.90	8.82	0.24	<0.01
Total	5.70	9.01	0.27	<0.01
Water excretion, L/d				
Feces	0.59	0.75	0.09	0.07
Urine	2.54	4.40	0.37	<0.01
Total	3.13	5.15	0.43	<0.01
Water balance, L/d	2.57	3.86	0.65	<0.01

<sup>1</sup> DMI, dry matter intake; ADMD, apparent dry matter digestibility.

<sup>2</sup> SEM, standard error of the mean.

<sup>3</sup> t-test P-value.

**Table 4.6** Nitrogen dynamics from information obtained from a 48-hr period within metabolism crates of Coopworth lambs fed either a monotonous diet (perennial ryegrass only, SF) or a taxonomically diverse multi-forage choice (MF) diet of equal proportions of fresh cut herbage of perennial ryegrass (*Lolium perenne* L.), alfalfa (*Medicago sativa* L.), chicory (*Cichorium intybus* L.), and plantain (*Plantago lanceolata* L.).

Item	Treatments		SEM <sup>1</sup>	P-value <sup>2</sup>
	SF	MF		
Nitrogen intake, g/d	36.05	54.56	1.68	<0.01
Apparent nitrogen digestibility, %	86.26	88.63	1.70	0.09
Urinary nitrogen concentration, g/L	6.27	4.39	0.41	<0.01
Nitrogen excretion, g/d				
Feces	5.09	6.06	0.92	0.13
Urine	15.66	17.83	2.50	0.26
Total	20.75	21.06	2.82	0.86
Apparent nitrogen retention, g/d	15.29	30.67	2.92	<0.01

<sup>1</sup>SEM, standard error of the mean.

<sup>2</sup> t-test P-value.

**Table 4.7** Rumen ammonia (NH<sub>3</sub>) and rumen volatile fatty acid (VFA) profile of ram lambs on days 20 and 35 fed either a monotonous diet (perennial ryegrass only, SF) or a taxonomically diverse MF diet of equal proportions of fresh cut herbage of perennial ryegrass (*Lolium perenne* L.), alfalfa (*Medicago sativa* L.), chicory (*Cichorium intybus* L.), and plantain (*Plantago lanceolata* L.).

Item <sup>1</sup>	Treatments		SEM <sup>2</sup>	P-value <sup>3</sup>
	SF	MF		
NH <sub>3</sub> , mmol/L	8.74	9.15	0.67	0.70
Total VFA, mmol/L	35.84	36.92	3.00	0.81
Ace:Prop	4.45	4.23	0.09	0.10
VFA profile, % of Total VFA				
Acetate	69.39	68.21	0.48	0.10
Propionate	15.62	16.30	0.33	0.17
Iso-butyrate	2.40	2.48	0.21	0.80
Butyrate	8.61	9.10	0.24	0.16
Iso-valerate	1.10	0.97	0.09	0.31
Valerate	0.09	1.06	<0.01	0.02

<sup>1</sup> NH<sub>3</sub>, ammonia; Total VFA, total volatile fatty acid; Ace:Prop acid, proportion of acetate to propionate.

<sup>2</sup> SEM = Standard error of the mean.

<sup>3</sup> t-test P-value.

Note: Hexanoic and lactic acid were not included as the amounts present were below the detection limit gas chromatogram.

**Table 4.8** Behavior within daylight hours of ram lambs on days 11 and 32 being fed either a monotonous diet (perennial ryegrass only, SF) or a taxonomically diverse MF diet of equal proportions of fresh cut herbage of perennial ryegrass (*Lolium perenne* L.), alfalfa (*Medicago sativa* L.), chicory (*Cichorium intybus* L.), and plantain (*Plantago lanceolata* L.).

Behavior, % of time	Treatments				P-value		
	SF		MF		TRT	Time	TRT×Time
	AM	PM	AM	PM			
Eating	35.29±1.27 <sup>c</sup>	47.28±1.61 <sup>b</sup>	37.34±1.27 <sup>c</sup>	64.44±2.19 <sup>a</sup>	<0.01	<0.01	0.01
Ruminating	21.64±2.09 <sup>ab</sup>	24.30±2.35 <sup>a</sup>	23.82±2.30 <sup>a</sup>	17.34±1.68 <sup>b</sup>	0.26	0.36	0.02
Idle	33.88±2.49	26.38±1.82	26.53±1.84	21.70±1.38	0.01	0.01	0.09
Stereotypic behavior	1.14±0.31 <sup>a</sup>	0.47±0.13 <sup>b</sup>	0.93±0.25 <sup>ab</sup>	0.19±0.05 <sup>c</sup>	0.59	<0.01	0.05
Groom	0.48±0.12	0.77±0.22	0.49±0.12	0.80±0.23	0.93	0.12	0.81
Observing SF	0.89±0.41	0.33±0.14	0.28±0.11	0.18±0.05	0.05	0.10	0.98
Observing MF	1.43±0.31	2.06±0.31	0.14±0.31	0.77±0.31	<0.01	0.15	0.54
Observing Humans	3.59±0.93	0.41±0.07	4.95±1.28	0.42±0.08	0.38	<0.01	0.28

<sup>a-c</sup> Means in a row without similar superscripts differ ( $P < 0.05$ ).

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## Chapter 5

# It is not just what is fed but how we serve it through time — a varied diet increases intake of lambs

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### 5.1 Abstract

The effect of repeated allocation of a single component diet on dry matter intake (DMI) is not fully understood. To determine if repeated feed allocation affected intake and rumen characteristics, 21 Coopworth rams were allocated to one of three treatments: repetition of ryegrass (*Lolium perenne* L.; RR), repetition of a mix (RM) of ryegrass, alfalfa (*Medicago sativa* L.), chicory (*Cichorium intybus* L.), or plantain (*Plantago lanceolata* L.) or varied diet (VD; selection from equal parts DM of two feeds: ryegrass, alfalfa, chicory, or plantain in the AM (0700 h to 1600 h), with the remaining two feeds allocated in the PM (1600 h to 0700 h). All plants by feeding time combinations were randomized by day for the VD. Diets were fed ad libitum for 38 days and intake was recorded. The VD improved DMI (20 and 10%, respectively) and reduced the day-to-day coefficient of variation of DMI (29 and 23%, respectively) compared with the RR and RM diets. The diet components of each of the diverse diets (RM and VD) resulted in a greater percentage of the rumen volatile fatty acids (VFAs) being comprised of butyrate and valerate. Further research is required to determine if this difference in DMI was the result of the ability to choose between different feeds or if it was the temporal variation in feed availability.

**Key words:** varied diets, intake, monotony, rumen

### 5.2 Introduction

Heraclitus's theory that a monotonous and unchanging state is an unnatural condition for living things may hold in terms of today's farmed generalist herbivores. Ancestors of today's

ruminants grazed in diverse and changing environments and selected diets from a range of biochemically diverse plant species (Provenza et al., 2007). Yet, diversity is not always incorporated into conventional pastoral systems which commonly offer a 'simple' single herbage or a mixed sward where feed choice and feeding decisions through time are often non-existent at the animal level. Such systems thereby may impose unnatural dietary restrictions on animals and the consequences of this are not understood fully. Studies such as those by Cosgrove et al. (2001), Villalba et al. (2011), and Garrett et al. (2021) have shown that animals given diversity, in terms of choice between several different feed options, can have improved intake, performance, and reduced environmental impacts relative to a single feed. Increased production in Garrett et al. (2021) from lambs offered a diverse diet (grass, two herbs and a legume) compared with those fed a ryegrass diet may have been due to differences in diet chemical composition alone or if a monotonous mixture of the diverse components could yield the same production benefits. Such dietary monotony can elicit the phenomenon of incidental restriction, where intake ceases as a particular nutrient surpasses its threshold or an animal's ability to negate its toxic effects (Raubenheimer, 1992; Villalba et al., 2015b; Gregorini et al., 2017). Reduced intake can occur with repetitive diet allocation as sensory-based aversion (sensory-specific satiety) is introduced and the response from gustatory, olfactory, and visual neurons is diminished due to the repetitive exposure (Bailey and Provenza, 2008; Provenza, 1995; Provenza, 1996)).

In addition, the temporal allocation of different feeds has an effect on intake (Papachristou et al., 2007; Jensen et al., 2013). Although, less information is known on the effect of altering the temporal availability of fresh herbages on intake compared with animals consuming a homogenous mix of the same dietary components. However, based on Heraclitus's theory, changing the availability of dietary options may provide a more natural condition and allow for improvements in intake relative to dietary repetition. We hypothesize that dietary variety will increase dry matter intake and alter rumen parameters (e.g. total VFA concentration) relative to a repetitive ryegrass or diverse mixed diet. The objective of this study was two-fold. Firstly, to compare a homogenized mixture of diverse diet (grass, herbs and legume) components to a monotonous ryegrass diet to see if the effects detected by Garrett et al. (2021) were due to biochemical composition of the diet allocated alone. Secondly, to compare the intake and rumen parameters of animals repeatedly fed a diet of ryegrass (RR), a repeated presentation of a homogenized mixture of diverse components (RM), or a diet in which combinations of feeds is varied over the course of a day (VD). Finally, comparing the VD and RM diet allowed us to determine if effects on intake are due to the ability to choose and variation in feed availability or simply the result of dietary composition.

## 5.3 Materials and Methods

The study was conducted at the Johnstone Memorial Laboratory at Lincoln University (43°38'57"S, 172°27'01"E), according to the methods approved by the Lincoln University Animal Ethics Committee (AEC 2019-17).

### 5.3.1 Animal Management and Dietary Treatments

Twenty-one 7-month old Coopworth rams ( $39.9 \pm 0.9$  kg initial live weight; Mean  $\pm$  SEM) were housed in individual pens indoors for 38 d starting on 12 April 2019. Prior to experiment initiation, all animals had been grazing ryegrass (*Lolium perenne* L.). Animals were randomly assigned to one of three treatments: repetition of ryegrass (RR), repetition of a homogenized mix (RM) of ryegrass, alfalfa (*Medicago sativa* L.), chicory (*Cichorium intybus* L.), or plantain (*Plantago lanceolata* L.), or varied diet (VD; selection from equal parts DM of two feeds: ryegrass, alfalfa, chicory, or plantain in the AM (0700 h to 1600 h), with the remaining feeds allocated in the PM (1600 h to 0700 h). All plants by feeding time combinations were randomized by day for the VD. Reference to sequences fed to the varied diet are referred to throughout the manuscript as follows: C = chicory, L = alfalfa, P = plantain, and R = ryegrass, and the two feeds appearing before the colon are those allocated in the AM and the two feeds following the colon are those allocated in the PM e.g., AM feeds: PM feeds. Both the RR and RM treatments received a fresh allocation of their respective diets at 1600 h, when the VD treatment was presented with their PM herbage options. Each animal had *ad libitum* access to their respective treatment diet, based on their previous day's orts. A Haldrup forage harvester (Haldrup GmbH, Ilshofen, Germany) was used to cut feed ~3 cm above ground level. Feed was stored in a chiller (4°C) until it was fed and kept for up to 2 days after harvest in case animals consumed more than was harvested on a given day. At each feeding time, the orts from the previous feeding were weighed. Samples of allocated and refused herbages were collected at each feeding. Each pen was cleaned daily and water was freely available to animals.

### 5.3.2 Animal Sampling and Measurements

Animals were sampled for blood and ruminal fluid on days 1, 20, and 38. A 10 mL sample of blood was obtained by jugular venipuncture into a heparinized blood tube (Greiner Bio-One International GmbH, Kremsmünster, Austria). A 2 mL subsample of the whole blood was stored at -20°C. The remaining blood was centrifuged (Megafuge 1.0R, Heraeus Holding GmbH, Hanau, Germany) at  $2,300 \times g$  and 4°C for 15 min, plasma was aspirated and stored at -20°C. Ruminal fluid was obtained via oesophageal tubing and sub-sampled into three 2 mL samples, one of which was acidified with sulphuric acid (10- $\mu$ L of 98% sulfuric acid; Fisher Scientific, Loughborough, United Kingdom).

Scan sampling at 2-min intervals was used to determine and record behaviors during daylight hours (0700-1734 h) on days 25-26 as described by Altmann (1974) and Villalba et al. (2015a). Artificial lighting was used within the facilities during this period. Behaviors recorded included: eating, ruminating, stereotypic behaviors (the sum of pacing and chewing pen fixtures, head butting pen fixtures, head hanging, crouching, pawing, and rearing), grooming (sum of scratching self and rubbing on pen fixtures), and observing humans (See Garrett et al., (2021) for an outline of each behavior and behavior selection criteria). On day 25 (observation d 1) the VD animals received ryegrass and alfalfa in the AM and chicory and plantain in the PM, and on day 26 (observation d 2) they received the same feed pairings in reverse order.

### 5.3.3 Sample Analysis

Freeze-dried herbage was ground by a centrifugal mill (ZM200; Retsch, Haan, Germany; 1 mm screen) and the chemical composition was determined using near-infrared spectrophotometry (NIRS; Model: FOSS NIRS Systems 5000, Maryland, USA see Garrett et al. (2021) for details on calibration equations). The metabolizable energy (ME) of herbages was estimated based on the Primary Industries Standing Committee, (2007) equation:

$$[ME (MJ/kg DM) = \text{digestible OM in DM, \%}(DOMD) \times 0.16] \quad [1].$$

Glutathione peroxidase (GPx) was determined in whole blood samples using an enzymatic based protocol (RANSEL; Cat. No. RS504) and a clinical analyzer (Randox Rx Daytona, Crumlin, Co. Antrim, UK). The total antioxidant status (TAS) of the plasma was determined using a colorimetric method on the clinical analyzer using a commercial kit (Cat. No. NX2332; Randox Rx Daytona, Crumlin, Co. Antrim, UK).

Ammonia concentration was determined in the acidified rumen samples using the clinical analyzer (Randox Rx Daytona, Crumlin, Co. Antrim, UK) and a commercial test kit (Cat. No. AM3979; Randox; Crumlin, Co. Antrim, UK) based on the enzymatic UV method described by Neeley and Phillipson (1988). Ruminal volatile fatty acid (VFA) concentration was determined using a gas chromatograph (Shimadzu GC-2010, Kyoto, Japan with AOC-20i auto-sampler) fitted with an SGE BP21 30 m × 530 μm × 1 μm wide-bore capillary column as described by (Chen and Lifschitz, 1989). A separate commercial separate kit (Cat. No. LC2389; Randox; Crumlin, Co. Antrim, UK) and the Randox Rx Daytona clinical analyser was used to determine the lactate concentration of the ruminal fluid.

### 5.3.4 Statistical Analysis

All statistical analyses were conducted using R (R Core Team, 2018, v.3.4.4). Data that were normally distributed ( $P > 0.10$ ; Shapiro-Wilk test) and that had homogenous variance ( $P > 0.10$ ;

Bartlett's test) were analyzed with an analysis of variance (ANOVA) using the 'aov' function of R (R Core Team, 2018). Non-normally distributed data were analyzed by a generalized linear model (GLM) using R's 'glm' function (R Core Team, 2018). The d 1 samples (pre-treatment) were explored as covariates for the rumen and blood variables and included in the model if they explained a significant ( $P < 0.05$ ) amount of variation. Differences in chemical composition between herbage and diets were tested using the 'lme' mixed model function, with day as a random effect.

Behavior data that were normally distributed (time eating and ruminating) were analyzed using the 'aov' function. Non-normally distributed data were analyzed by GLM using the 'glm' function of R, with the distribution used for the model selected based on qq-plots of the residuals. The model for the animal behavior included the treatment, observation time (morning = 0700 – 1200 h; afternoon = 1200 – 1734 h), and their interaction as fixed effects. Upon significance of the ANOVA, means separation between diets were done using a pairwise t-test using the 'emmeans' package (Lenth, 2018). Statistical significance was declared at  $P \leq 0.05$  with tendencies declared at  $0.05 < P \leq 0.10$ .

## 5.4 Results

### 5.4.1 Diet Composition

The chemical composition of each herbage comprising the diets offered to both treatments is presented in Table 5.1. These values were used to calculate the average chemical composition of each diet, which are compared in Table 5.2. The proportions of chicory (Mean  $\pm$  SEM; RM =  $26.84 \pm 0.76\%$ , VD =  $26.71 \pm 0.76\%$ ), ryegrass (RM =  $21.72 \pm 0.67\%$ , VD =  $21.81 \pm 0.67\%$ ), alfalfa (RM =  $22.18 \pm 1.06\%$ , VD =  $21.80 \pm 1.03\%$ ), and plantain (RM =  $23.31 \pm 0.84\%$ , VD =  $23.78 \pm 0.87\%$ ) comprising each of the diets allocated to the RM and VD were not different ( $P > 0.10$ ). The ME content ( $P = 0.14$ ) and the dry matter content ( $P = 0.48$ ) of all the diets did not differ. The RM and VD diets did not differ in chemical composition ( $P > 0.10$ ). The organic matter (OM), Neutral Detergent Fiber (NDF), Acid Detergent Fiber (ADF), and Crude Protein (CP) content of the RR diet were all greater ( $P < 0.01$ ) than the RM and VD diets. The Organic Matter Digestibility (OMD;  $P = 0.05$ ) and Water Soluble Carbohydrate (WSC;  $P = 0.01$ ) content of the RR diet allocated were less than that of the RM and VD diets. The only difference in the botanical composition of the allocated and refused RM diet was a greater proportion of dead material ( $36.6 \pm 0.09\%$ ) in the refused material compared with the allocated ( $11.5 \pm 0.09\%$ ;  $P < 0.02$ ), indicating that RM fed lambs were unable to sort specific forage species from the mixed diet.

### 5.4.2 Intake

Intake by VD lambs was 20% and 10% greater ( $P < 0.01$ ; Table 5.3) compared with the RR and RM, respectively. Lambs in RM had greater Dry Matter Intake (DMI) (10%) than RR lambs ( $P < 0.05$ ). The DMI for the AM and PM had the same trend in DMI, increasing from RR, to RM, to VD, with statistical differences between each treatment at each time ( $P < 0.05$ ). The within animal day-to-day coefficient of variation (CV) of DMI was 42% and 30% greater ( $P < 0.05$ ) for the RR and RM compared with the VD, respectively. There was no difference in the CV of DMI between RR and RM ( $P > 0.05$ ), although the RR CV of DMI was numerically (9%) greater. The order and combinations of feeds allocated to the VD resulted in differences in DMI in the AM, PM, and over the whole day ( $P < 0.01$ ; Table 5.4). The intake of the VD rams was greater when they consumed the CL:PR and CR:LP sequences, compared with the CP:LR and LR:CP ( $P < 0.05$ ), while LP:CR and PR:CL were intermediate, and not different to the other combinations ( $P > 0.10$ ).

### 5.4.3 Behavior

In the afternoon, the lambs in VD spent 11% more time eating compared with the lambs in RM ( $P < 0.05$ ; Table 5.6). Both the RR and RM lambs spent a greater percentage of time eating in the morning than in the afternoon ( $P < 0.05$ ). There was a tendency for an interaction between treatment  $\times$  time of day for the percent of time spent conducting stereotypic behaviors ( $P = 0.10$ ), with the RM lambs conducting more stereotypic behaviors in the afternoon, compared with the morning. The lambs in RM also tended to exhibit more stereotypic behaviors in the afternoon, compared with lambs in RR.

### 5.4.4 Blood and Rumen Parameters

Levels of GPx in plasma were not different ( $P = 0.43$ ) between lambs in VD ( $22,584 \pm 1,586$  U/L), RR ( $20,598 \pm 1,618$  U/L), and RM ( $23,559 \pm 1,750$  U/L). However, there was a treatment effect ( $P = 0.05$ ) for TAS, with lambs in RM ( $1.09 \pm 0.06$  mmol/L) having a lower ( $P \leq 0.05$ ) TAS than lambs in RR ( $1.31 \pm 0.08$  mmol/L). The VD treatment TAS ( $1.24 \pm 0.07$  mmol/L) was intermediate and not different ( $P > 0.10$ ) to either the RM or RR. There was a treatment effect on the percent of the VFA profile composed of butyrate ( $P = 0.03$ ; Table 5.5) and valerate ( $P = 0.02$ ). The percentage of the VFA profile accounted for by butyrate and valerate was lower for the RR compared with the RM and VD lambs, while RM and VD lambs were not different ( $P > 0.05$ ) to one another.

## 5.5 Discussion

Overall the results partially support the hypothesis that dietary variety will stimulate lambs to eat more and alter their rumen fermentation pattern relative to repetitive allocations of ryegrass or

even a diverse mixed diet. However, the effects of rumen parameters were slight, with only valerate and butyrate percentages differing between treatments; however, time of sampling (once daily prior to feeding) may have influenced these results.

The VD and RM lambs had greater DMI than RR lambs, suggesting that differences in the chemical composition of the diet are partly responsible for the greater DMI of these diets. This is supported by the greater NDF content of the RR diet, which has been associated with reduced DMI due to physical constraints on intake as rumen retention time is increased (Mertens, 1994). This was largely expected as grasses, such as ryegrass, typically have more girder structures (structural) cells, which to reduce forage digestibility relative to other forages (e.g. herbs and legumes) (Wilson and Kennedy, 1996). Although, it is noteworthy that the VD and RM diets did not have the same DMI, allowing the suggestion that the greater DMI of VD relative to RM may have been a result of lambs having the ability to choose and combine different nutrients and oro-sensorial experiences over time thereby avoiding nutrient and sensory-specific satiety provided by the monotonous mix (Beck and Gregorini, 2021; Garrett et al., 2021). Increased DMI of treatments offered biochemical or flavorally diverse diets compared with a monotonous one has been reported in lambs fed grain and silage-based diets (Distel et al., 2007; Villalba et al., 2011), conserved forages (Distel et al., 2007), and also fresh forages (Garrett et al., 2021). While the temporal feed allocation of different feeds is used by French herders to design feeding circuits to stimulate feeding motivation and optimize use of diverse forages (Meuret and Provenza 2015). Having greater DMI when provided varied choice-based diet indicates that how the diets are presented to the animal affects intake in addition to the chemical composition. Further research is required to determine if the effect on DMI is a result of the animal's ability to choose or if it was an effect of the varied dietary components available.

The VD treatment had a more consistent DMI compared with the RM and RR. A more consistent DMI was also reported by Garrett et al. (2021) with lambs offered choice between set ratios of a diverse herbage diet components having a 26% lower day-to-day DMI CV than lambs offered 100% ryegrass. Further, Garrett et al. (2021) reported a negative correlation ( $r = -0.60$ ) between CV of DMI and average daily gain, indicating that increases in performance could be expected by the VD dietary treatment within the present study. Further, the order of availability of feeds throughout the day affected the DM consumption by the VD lambs. Ingestion sequence influences intake of pelleted, grain, and alfalfa diets (Papachristou et al., 2007; Mote et al., 2008; Jensen et al., 2013). For example, Mote et al. (2008) determined that intake tended to be greater when feed was allocated in the order of tannins, terpenes, and alfalfa/barley. Within the present study, some feed combinations may have provided non-complementary flavors or plant secondary compound combinations. Alternatively, they may have resulted in ingestion of plant primary or secondary compounds requiring the same detoxification system, with only two feeds to select from



sheep would have been unable to negate such effects and reduced feed intake to prevent further effects. The feed combination of alfalfa and ryegrass at one time point (i.e., AM or PM), and chicory and plantain at the other resulted in the numerically lowest total intake. Ryegrass and alfalfa both have high concentrations of crude protein and the resulting high concentrations of rumen ammonia from such high protein diets can cause food aversions (Provenza, 1995). While chicory and plantain shared more similarities in chemical profile than with other feeds. Translation of the 'optimum' sequences and the results from the present study to a practical setting should be interpreted cautiously, as all forages were harvested in the morning and it is well documented that herbage chemical composition (i.e. increased WSC content of pastures in the afternoon) and digestibility varies throughout the day (Gregorini, 2012). Thereby, forage harvested or grazed at a different time of day may produce different results from the present study and further investigation using different forage harvesting times is required.

In the afternoon, the VD rams spent more time eating than the RM rams, indicating that the method of presentation and perhaps choice from diverse diet components can alter intake patterns. The study by Garrett et al. (2021) comparing rams eating ryegrass with a diverse diet, reported rams consuming diverse diets spent a greater percentage of their time eating. However, this difference was not seen between the VD and RR. Thereby, further investigation is required on how ingestive behavior is affected by feeding sequences.

Both the VD and RM diets total VFA profile contained a greater percentage of butyrate, which in ruminants is primarily an energy source for epithelial cells and stimulates their proliferation, improving feed utilization by animals (Miguel et al., 2019). This implies that, in addition to greater intake compared with the RR, both the VD and RM could have greater feed utilization. However, further investigation is required. Similarly, the VD and RM diets total VFA profile contained a greater percentage of valerate, which like propionate requires H<sub>2</sub> to produce, reducing the amount of H<sub>2</sub> available for methane production (a potent greenhouse gas) (Jonker et al., 2019). In addition, the formation of butyrate is associated with the production of less H<sub>2</sub> than other VFAs like acetate (Jonker et al., 2019). These results provide limited evidence that a set ratio VD and RM diet could reduce methane emissions; however, further research is required.

## **5.6 Conclusions**

Lambs provided with a varied diet increased DMI and reduced the day-to-day coefficient of variation of DMI relative to a repetitive diet of ryegrass or a mixture of the same components present in the varied diet, over and above that which can be explained by dietary composition alone. Further, the diverse diet components (RM and VD) altered fermentation leading to greater percentage of the rumen VFA being comprised by butyrate and valerate. Further research is required

to determine if this difference in DMI was the result of VD ram's ability to choose between different feeds, or if it was the temporal variation in feed availability.

**Table 5.1** Chemical Composition of the herbage composing the total diets offered.

Item <sup>2</sup>	Herbage				SEM <sup>1</sup>
	Chicory	Alfalfa	Plantain	Ryegrass	
ME, MJ/kg DM	12.4 <sup>a</sup>	10.8 <sup>b</sup>	11.7 <sup>ab</sup>	12.1 <sup>ab</sup>	0.7
DM, % as-is	8.6 <sup>d</sup>	16.2 <sup>a</sup>	10.1 <sup>c</sup>	12.3 <sup>b</sup>	0.5
DMD <sub>p</sub>	86.0 <sup>a</sup>	63.6 <sup>d</sup>	80.8 <sup>b</sup>	75.3 <sup>c</sup>	1.6
OM	86.7 <sup>c</sup>	91.3 <sup>a</sup>	86.4 <sup>c</sup>	89.4 <sup>b</sup>	0.5
OMD	91.3 <sup>a</sup>	65.0 <sup>d</sup>	85.2 <sup>b</sup>	79.1 <sup>c</sup>	1.6
WSC	24.2 <sup>a</sup>	5.3 <sup>b</sup>	26.9 <sup>a</sup>	9.2 <sup>b</sup>	2.6
NDF	17.6 <sup>d</sup>	37.7 <sup>b</sup>	22.6 <sup>c</sup>	46.8 <sup>a</sup>	1.2
ADF	18.6 <sup>d</sup>	30.3 <sup>a</sup>	20.7 <sup>c</sup>	27.8 <sup>a</sup>	0.8
CP	19.9 <sup>b</sup>	23.2 <sup>a</sup>	16.1 <sup>c</sup>	22.3 <sup>a</sup>	0.9

<sup>a-d</sup> Means in a row without similar superscripts differ ( $P \leq 0.05$ ).

<sup>1</sup> SEM = standard error of the mean.

<sup>2</sup> ME = metabolizable energy; DM = dry matter; OM = organic matter; OMD = OM digestibility; WSC = water soluble carbohydrates; NDF = neutral detergent fiber; ADF = acid detergent fiber; CP = crude protein

**Table 5.2** The chemical composition of the repetitive ryegrass (RR) diet and the calculated chemical composition of the varied (VD) and repetitive mix (RM) diet offered to the ram lambs<sup>1</sup>

Item <sup>1</sup>	Treatment Diet <sup>2</sup>			SEM <sup>3</sup>	P <sup>4</sup>
	RR	RM	VD		
ME, MJ/kg DM	12.10	11.84	11.84	0.14	0.14
DM, % DM	12.25	12.23	12.23	0.19	0.48
OM, % DM	89.37 <sup>a</sup>	88.21 <sup>b</sup>	88.18 <sup>b</sup>	0.18	<0.01
OMD, % OM	79.10 <sup>b</sup>	80.56 <sup>a</sup>	80.65 <sup>a</sup>	0.60	0.05
WSC, % DM	9.19 <sup>b</sup>	16.45 <sup>a</sup>	16.65 <sup>a</sup>	0.28	0.01
NDF, % DM	47.77 <sup>a</sup>	30.44 <sup>b</sup>	30.24 <sup>b</sup>	0.76	<0.01
ADF, % DM	27.79 <sup>a</sup>	24.24 <sup>b</sup>	24.17 <sup>b</sup>	0.14	<0.01
CP, % DM	22.34 <sup>a</sup>	20.43 <sup>b</sup>	20.35 <sup>b</sup>	0.08	<0.01

<sup>a-b</sup> Means in a row without similar superscripts differ ( $P \leq 0.05$ ).

<sup>1</sup> ME = metabolizable energy; DM = dry matter; OM = organic matter; OMD = OM digestibility; WSC = water soluble carbohydrates; NDF = neutral detergent fiber; ADF = acid detergent fiber; CP = crude protein.

<sup>2</sup> Values for diverse composition were calculated by using the percentage of the item value that each dietary component accounted for. RM = repetitive presentation of a homogenously mixed diet of equal parts chicory, ryegrass, plantain, and alfalfa; VD = Varied diet, selection from equal parts DM of two feeds: ryegrass, alfalfa, chicory, or plantain in the AM, with the remaining feeds allocated in the PM.

<sup>3</sup> SEM = Standard error of the mean.

<sup>4</sup> P = t-test P-value.

**Table 5.3** Mean dry matter intake (DMI) and day-to-day-DMI co-efficient of variation (DMI CV) of ram lambs fed a varied (VD), repetitive mix (RM), and repetitive ryegrass (RR) diet over 38 days.

Item <sup>1*</sup>	Treatment <sup>2</sup>			SEM <sup>3</sup>	P <sup>4</sup>
	RR	RM	VD		
DMI, kg/d	1.00 <sup>c</sup>	1.10 <sup>b</sup>	1.20 <sup>a</sup>	0.01	<0.01
AM DMI, kg/d	0.48 <sup>c</sup>	0.54 <sup>b</sup>	0.60 <sup>a</sup>	0.01	<0.01
PM DMI, kg/d	0.52 <sup>c</sup>	0.56 <sup>b</sup>	0.60 <sup>a</sup>	0.02	<0.01
DMI CV, %	17.48 <sup>a</sup>	15.98 <sup>a</sup>	12.32 <sup>b</sup>	1.17	<0.01

<sup>1</sup> AM DMI = DMI from 0700 – 1600h; PM DMI = DMI from 1600 – 0700 h

<sup>2</sup> RR = repetitive diet of ryegrass; RM = repetitive diet of a homogenous mixture of equal parts chicory, ryegrass, plantain, and alfalfa; VD = Varied diet, selection from equal parts DM of two feeds: ryegrass, alfalfa, chicory, or plantain in the AM, with the remaining feeds allocated in the PM.

<sup>3</sup> SEM = Standard error of the mean.

<sup>4</sup> P = t-test P-value.

<sup>a-c</sup> Different superscripts (a-c) within a row indicate significant differences at  $P \leq 0.05$ .

**Table 5.4** Comparison of dry matter intake with different orders of feed presentation for the varied diet (VD)

	Feeding sequence <sup>1</sup>						SEM <sup>3</sup>	P-value <sup>4</sup>
	CL:PR	CP:LR	CR:LP	LP:CR	LR:CP	PR:CL		
n <sup>2</sup>	6	7	5	6	8	6		
AM DMI	0.62 <sup>a</sup>	0.59 <sup>ab</sup>	0.67 <sup>a</sup>	0.57 <sup>ab</sup>	0.52 <sup>b</sup>	0.58 <sup>a</sup>	0.04	<0.01
PM DMI	0.62 <sup>a</sup>	0.56 <sup>b</sup>	0.58 <sup>ab</sup>	0.62 <sup>a</sup>	0.62 <sup>a</sup>	0.62 <sup>a</sup>	0.02	<0.01
Total DMI, kgd <sup>-1</sup>	1.26 <sup>a</sup>	1.15 <sup>b</sup>	1.27 <sup>a</sup>	1.21 <sup>ab</sup>	1.16 <sup>b</sup>	1.19 <sup>ab</sup>	0.04	<0.01

<sup>1</sup> C = Chicory, L = Alfalfa, P = Plantain, R = Ryegrass, the two feeds appearing before the colon are those allocated in the AM (0700 to 1600 h) and the two feeds following the colon are those allocated in the PM (1600 to 0700 h) eg. AM feeds:PM feeds.

<sup>2</sup> n = number of times that combination of feed was allocated.

<sup>3</sup>SEM = Standard error of the mean.

<sup>4</sup> t-test P-value.

<sup>a-b</sup> Different superscripts (a-b) within a row indicate significant differences at  $P \leq 0.05$ .

**Table 5.5** Rumen ammonia (NH<sub>3</sub>) and rumen volatile fatty acid (VFA) profile of ram lambs on d20 and 38 fed a repetitive diet of ryegrass (RR), a repetitive diet of homogenously mixed equal parts chicory, plantain, alfalfa, or ryegrass (RM), or a varied diet with selection from equal parts DM of two feeds: ryegrass, chicory, plantain, and alfalfa in the AM (0700 to 1600 h), with the remaining two feeds allocated in the PM (1600 to 0700 h)

Item <sup>1</sup>	Treatments			SEM <sup>2</sup>	P-value <sup>3</sup>
	RR	RM	VD		
NH <sub>3</sub> , mmol/L	9.12	10.46	9.72	1.00	0.56
Total VFA, mmol/L	31.97	32.32	31.05	2.65	0.56
Ace:Prop	4.41	4.51	4.39	0.13	0.91
VFA profile, % of total					
Acetate	66.95	66.37	66.46	0.05	0.68
Propionate	15.21	14.86	15.24	0.39	0.68
Iso-butyrate	2.16	2.36	2.69	0.35	0.51
Butyrate	10.77 <sup>b</sup>	11.67 <sup>a</sup>	11.67 <sup>a</sup>	0.27	0.03
Iso-valerate	2.71	2.63	2.60	0.10	0.85
Valerate	1.02 <sup>b</sup>	1.14 <sup>a</sup>	1.14 <sup>a</sup>	0.04	0.02

<sup>a-b</sup> Means in a row without similar superscripts differ ( $P < 0.05$ ).

<sup>1</sup>NH<sub>3</sub> = ammonia; Total VFA = total volatile fatty acid; Ace:Prop acid = proportion of acetate to propionate.

<sup>2</sup>SEM = Standard error of the mean.

<sup>3</sup>t-test  $P$ -value.

Note: Hexanoic and lactic acid were not included quantities were below the detection limit.

**Table 5.6** Behavior within daylight hours of ram lambs on day 25 and 26 being fed either a repetitive diet of ryegrass (RR) or a taxonomically diverse multi-forage mix (RM) diet of equal proportions of fresh cut herbage of perennial ryegrass (*Lolium perenne* L.), alfalfa (*Medicago sativa* L.), chicory (*Cichorium intybus* L.), and plantain (*Plantago lanceolata* L.), or a varied diet with selection from equal parts DM of two feeds: ryegrass, chicory, plantain, and alfalfa in the AM (0700 to 1600 h), with the remaining two feeds allocated in the PM (1600 to 0700 h). The values reported in this table are least-squares means  $\pm$  the standard error of the mean for the proportion of time spent doing a specific behavior. Morning = 0700 – 1200 h and afternoon = 1200- 1734 h.

Behavior, % of time	Treatments						P-value		
	RR		RM		VD		TRT	Time	TRT×Time
	Morning	Afternoon	Morning	Afternoon	Morning	Afternoon			
Eating	40.38 $\pm$ 3.62 <sup>a</sup>	27.54 $\pm$ 2.47 <sup>bc</sup>	42.00 $\pm$ 3.77 <sup>a</sup>	20.53 $\pm$ 1.84 <sup>c</sup>	29.33 $\pm$ 2.63 <sup>a</sup> <sub>bc</sub>	31.69 $\pm$ 2.84 <sup>a</sup> <sub>b</sub>	0.46	<0.01	<0.01
Ruminating	22.88 $\pm$ 1.93	26.21 $\pm$ 2.34	21.06 $\pm$ 1.73	23.85 $\pm$ 2.08	18.21 $\pm$ 1.4 <sub>4</sub>	20.26 $\pm$ 1.7 <sub>1</sub>	0.06	0.15	0.74
Idle	35.86 $\pm$ 2.67	38.41 $\pm$ 2.86	32.33 $\pm$ 2.41	44.91 $\pm$ 3.35	39.29 $\pm$ 2.9 <sub>3</sub>	43.58 $\pm$ 3.2 <sub>5</sub>	0.34	<0.01	0.16
Stereotypic behavior	1.11 $\pm$ 0.40	0.51 $\pm$ 0.19	1.05 $\pm$ 0.37	2.63 $\pm$ 0.94	0.71 $\pm$ 0.26	0.89 $\pm$ 0.32	0.64	0.07	0.10
Groom	1.02 $\pm$ 0.32	0.81 $\pm$ 0.26	0.57 $\pm$ 0.26	0.94 $\pm$ 0.30	0.72 $\pm$ 0.23	1.11 $\pm$ 0.35	0.79	0.41	0.41
Observing Human	1.38 $\pm$ 0.55	3.21 $\pm$ 1.29	1.95 $\pm$ 0.78	4.26 $\pm$ 1.71	2.52 $\pm$ 1.01	0.90 $\pm$ 0.36	0.35	0.29	0.05

<sup>a-c</sup> Means in a row without similar superscripts differ ( $P < 0.05$ ).



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## Chapter 6

# Varied diets: Implications for lamb performance, rumen characteristics, total antioxidant status, and welfare

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### 6.1 Abstract

Intensive pastoral systems have moved away from diverse and varied diets towards overly simple monotonous diets. Feed choice through time is an obsolete way of providing forage to animals, as intensive management schemes generally allocate a single herbage or a dyad mixed sward. Monotonous feeding regimes impose nutritional repetition, which may impair animal performance and welfare. The objective of this experiment was to determine the impact of a diverse diet [DIV; free choice from perennial ryegrass (*Lolium perenne* L.), plantain (*Plantago lanceolata* L.), alfalfa (*Medicago sativa* L.), and chicory (*Cichorium intybus* L.) at all times], a varied diet [VAR; choice from ryegrass and plantain in the AM (0700 – 1600 h), and chicory and alfalfa in PM (1600 – 0700 h)], and a single forage diet of alfalfa [SFA; alfalfa at all times], on DMI, performance, and welfare of lambs. Six-month-old Coopworth ram lambs (n=21) were offered their respective fresh-forage treatment (n = 7) diet indoors for 20 days. The DIV lambs consumed  $1.64 \pm 0.03$  kg DM/d (mean  $\pm$  SEM), which was 6% more ( $P < 0.05$ ;  $1.54 \pm 0.03$  kg DM/d) than the SFA and were not different ( $P > 0.05$ ;  $1.59 \pm 0.03$  kg DM/d) to the VAR lambs. Average daily gain (ADG) of DIV (296 g/d) and VAR (378 g/d) was 30 and 67 % greater ( $P < 0.05$ ) compared with the SFA lambs (227 g/d), respectively. The VAR lambs had 28% greater ( $P < 0.05$ ) ADG than the DIV lambs. Differences among treatments were detected ( $P < 0.05$ ) for the proportion of the day spent conducting the following behaviors: eating, ruminating, idling, lying, and standing. In addition, the number of bouts of stereotypic behaviors recorded from the SFA lambs ( $13.2 \pm 2.2$ ) was 150% greater ( $P < 0.05$ ) than the DIV ( $5.1 \pm 1.0$ ) and VAR ( $5.5 \pm 1.0$ ) lambs. Our results suggest that the varied diet offered can improve animal performance and welfare compared to a monotonous SFA diet. Feeding management to provide a varied diet can improve performance

relative to giving lambs free choice from taxonomically diverse forage options. Moreover, performance is affected by more than the primary chemical composition of the diet consumed, but how the diet is presented through time and the herbage species and quantities of each that are consumed to reach that chemical composition.

**Key words:** alfalfa, diverse, monotony, varied, sheep

## 6.2 Introduction

Ancestors of today's ruminants selected from a range of biochemically diverse plant species within their given foodscape, of which species availability, abundance, and chemical composition changed over time and space (Provenza et al., 2007). As such diversity is multifaceted and encompasses a) species or component richness, b) the abundance of each of the given species or component, c) how the species or component are distributed through space, d) the individuality within species (e.g. genotypic variation and resource utilization, and how a-d vary through the temporal scale (Tilman et al., 1997, Purvis and Hector, 2000; Mason et al., 2005; Mouillot et al., 2005). Due to the term diversity encompassing the availability of feeds at a given site and how they vary through time, we propose for the purpose of this paper that a **diverse** diet will describe the availability of feeds at a given site and that a **varied** diet will describe changing feed availability through time (within the day scale). Varying diet availability can be used as a grazing management tool and is being implemented successfully in extensive pastoral systems to increase animal performance and enhance ecosystems health (Meuret and Provenza, 2015). Intensive pastoral systems though, have turned away from diverse and varied diets towards repeated allocation (monotony) of simple diets where animals do not get to make a feed choice, as single or dyad mix sward favor easier, less complex practical implementation and management.

Monotonous feeding environments impose nutritional repetition, which may impair animal performance and welfare. Monotonous diet presentation reduces intake or productivity relative to animals provided choice from a diverse diet (Provenza et al., 2007; Rodríguez et al., 2007; Dixon and Pasinetti, 2010; Garrett et al., 2021). This can be the result of nutrient specific satiation, as the upper threshold for a specific nutrient is reached despite deficiencies in other nutrients existing (Raubenheimer, 1992; Early and Provenza, 1998; Gregorini et al., 2017), or sensory-specific satiety, where the repeated oro-sensorial experience (i.e. taste) saturates the intake-related sensorial neurons and reduces the response for a particular feed (Early and Provenza, 1998; Epstein et al., 2009; Gregorini et al., 2017). On the other hand, diverse diets that allow selectivity, enable individual animals to choose from feeds of differing nutrient and oro-sensorial profiles and can result in improved intake or performance (Villalba and Provenza, 1997; Papachristou et al., 2007; Rodríguez et al., 2007; Mote et al., 2008; Catanese et al., 2013; Garrett et al., 2021). In addition, varied diets can

also improve DMI relative to repeated allocation of a single feed and specific sequences of diet allocation can improve intake relative to other sequences (Mote et al., 2008; Jensen et al., 2013). For example, Mote et al. (2008) reported that offering sheep feed rich in tannins before a feed rich in terpenes doubled intake compared with a meal offered in the reverse order. Although there is information regarding increased DMI when feeding a range or specific sequences of plant secondary compounds (PSC; e.g. tannins and terpenes), less information is known on the effect of varied diets of fresh forages can have on the DMI, performance, and welfare of animals compared with those consuming a diverse diet of the same components or a single monotonous diet.

We hypothesize that a varied diet of fresh forages over the day will improve intake, performance, and welfare of lambs compared to dietary monotony and dietary diversity of the same herbage species on offer at the same time, all day. We also hypothesize that a diverse diet of fresh forages over the day will improve intake, performance, and welfare of lambs compared to single forage monotony. As a result, our objective was to compare the DMI, ADG, and welfare of lambs fed a monotonous single forage diet of alfalfa (SFA), choice from diverse (DIV) diet components ryegrass (*Lolium perenne L.*), plantain (*Plantago lanceolata L.*), alfalfa (*Medicago sativa L.*), and chicory (*Cichorium intybus L.*), and a varied (VAR) diet comprised of the diverse diet components offered in a sequence through time. Alfalfa was chosen to be fed in monotony and to be compare against the DIV and VAR diets as it is often used within New Zealand farming systems as a specialty finishing diet due to its ability to provide large amounts of high quality forage (Brown et al., 2000; Avery et al., 2008; Anderson et al., 2014; Moot et al., 2019). The chosen diverse multi-forage diet increases DMI and ADG relative to a monotonous ryegrass diet (Garrett et al., 2021). Comparing this diverse diet to another species, known as a high-performing and quality diet for finishing lambs in dryland settings, will allow us to determine if the effects reported in previous work from our laboratory were diet specific. Further, comparing the VAR and DIV diet will allow us to determine if a temporal approach to grazing management can improve performance relative to animals with free choice from the same species.

## **6.3 Materials and Methods**

This study was conducted at the Lincoln University Johnstone Memorial Laboratory (43°38'57"S, 172°27'01"E), as per the methods approved by the Lincoln University Animal Ethics Committee (#2019-33A).

### **6.3.1 Animal Management and Dietary Treatments**

Six-month-old Coopworth rams (n= 21) with an average live weight (LW) of 33.55 ± 0.51 kg (mean ± SEM) were housed indoors in individual pens for 20 days starting on 3 March 2020. Animals

were randomly allocated to one of three treatments: SFA [monotony of alfalfa], DIV [free choice of ryegrass, alfalfa, chicory, and plantain] or VAR [selection from ryegrass and plantain in the AM (0700 h to 1600 h) and selection from alfalfa and chicory in the PM (1600 h to 0700 h)]. The sequence was selected as it was one where lambs performed better in terms of DMI within a trial by Garrett et al. (Unpublished) where animals received all possible feeding combination. Further, this sequence provided animals with access to a legume for the greatest proportion of their time. When offered the choice ruminants have a greater partial preference towards legumes, allocating on average 70% of their time to grazing legumes in a grass or legume choice scenario (Rutter, 2010). Prior to experiment initiation all animals had been grazing alfalfa and had been reared together, thereby had the same early life dietary experience. Animals in the DIV diets were presented all four feeds in individual bins simultaneously, with two feeds placed at each end of the pen. The diet options available to the VAR animals at a given time were presented simultaneously, with one forage option available from bins at each end of the pen. The end of the pen that each forage occupied was randomly assigned for each pen and maintained for the duration of the trial.

All treatments were offered fresh forage daily at 0700 h and pens were cleaned prior to forage allocation. The VAR treatment was presented their PM options and the AM options were removed at 1600 h. Lambs on the SFA and DIV diets were also presented a PM diet allocation of their respective diets at 1600 h to eliminate any frequency of feed presentation effects. Each sheep had ad libitum access to their allocated treatment diet and fresh water. Orts from the previous feeding were weighed at 0700 and 1600 h prior to the allocation of fresh feed.

### **6.3.2 Herbage Composition, Establishment, and Harvesting**

Planting preparation included defoliation of existing herbages and application of glyphosphate (Weedmaster Ts540; 4 L/ha), fluroxypyr (Starane Xtra Herbicide; 1 L/ha), Carfentrazone-E (Hammer Force; 0.1 L/ha), and Polyalkyleneoxide (Slikka; 0.15 L/ha). Seven days after spraying the area was ploughed and power harrowed. The areas planted with alfalfa, and chicory had Trifluraline (2 lts/ha) applied and incorporated appropriately. On the 26 October 2019, a direct drill calibrated to each forage species with 7.6 cm row spacing was used to plant each species as a monoculture in spatially separated strips. Seeding rate was 25, 12, 16, and 14 kg/ha for ryegrass (cv. Legion), chicory (cv. Choice), alfalfa (cv. Titan), and plantain (cv. Agritonic), respectively. The established plantain and ryegrass was treated with Dicamba (Kamba 500; 0.4 L/ha) and application of Flumetsulum (Preside; 60g/ha) and mineral oil (Uptake; 1 L/ha) occurred for chicory, clover, and alfalfa pastures. The area was fertilized with 250 kg di ammonium phosphate.

Fresh herbage was cut daily ~3 cm above ground level with a Haldrup forage harvester (Haldrup GmbH, Ilshofen, Germany). Feed was otherwise fed whole and un-cut. Once cut, feed was

stored in a walk-in refrigerator (4 °C) until it was allocated, unutilized feed was disposed of within 2 days post-harvest. Feed stored for more than a day was kept for topping up herbage if the fresh forage from the relevant day ran short. Samples of allocated and refused herbage were taken at each feeding to determine the feed quality and DM consumed.

Herbage chemical composition of the individual species included in the diets are presented in Table 6.1, and the average chemical composition of the diets consumed is presented in Table 6.2. Chicory and alfalfa were all in a vegetative state, while plantain and ryegrass contained 19.0 and 6.9% stem respectively. The extended shoot leaf height of the chicory, alfalfa, plantain, and ryegrass were  $26.6 \pm 2.6$ ,  $50.2 \pm 2.4$ ,  $32.0 \pm 2.9$ , and  $21.9 \pm 2.9$  cm, respectively.

### **6.3.3 Animal Sampling and Measurements**

On days 13 and 18 a blood samples were collected at [09:30 h (0 h), 15:30 h (6 h), and 21:30 h (12 h)] to determine total antioxidant status (TAS) and glutathione peroxidase (GPx) concentration. Blood samples were obtained via jugular venipuncture and collected in 10 ml lithium heparinized blood tubes (Greiner Bio-One International GmbH, Kremsmünster, Austria). Whole blood subsamples were collected, plasma samples were collected by centrifuging (Megafuge 1.0R, Heraeus Holding GmbH, Hanau, Germany) the remaining sample at  $2,300 \times g$  and 4 °C for 15 min, and samples were then stored at -20 °C until analysis. Rumen fluid was obtained via esophageal tubing on days 1 and 17, an hour after the allocation of feed during the AM and PM, to allow comparison of rumen characteristics (e.g. ammonia concentration). Rumen samples were sub-sampled into three 2 mL Eppendorf tubes, one acidified with sulphuric acid (10- $\mu$ L of 98% sulfuric acid; Fisher Scientific, Loughborough, United Kingdom) and two without. Animals live weights were measured every 5 days, before the morning feed allocation. Average daily gain (ADG) was estimated for each individual animal by regression and feed conversion efficiency (FCE; g ADG/kg DMI) was calculated.

Trained observers conducted behavioral observations during daylight hours on days 9 (0700-2010 h) and 20 (0734 – 1942 h). During daylight hours throughout the trial artificial lighting was used. Observers scan sampled (Altmann, 1974; Villalba et al., 2015), recording the behavior of each animal every 2 mins. An ethogram of the behaviors is presented (Table 6.3). The behaviors recorded were based on previous studies (Done-Currie et al., 1984; Lauber et al., 2012; Catanese et al., 2013): eating (consumption of which feed was specified for VAR and DIV animals), ruminating, idle and position of the animal (standing or lying down) was recorded. In addition to the scan samples, observers also documented the occurrences of stereotypic behaviors, which are repeated behaviors with no apparent function and are indicative of poor welfare (Broom, 1991; Catanese et al., 2013), and grooming behaviors. Stereotypic behaviors were considered to be the sum of incidences of pacing,



chewing, head butting, head hanging, pawing or stamping, rearing, or crouching (cowering). Grooming was considered as the sum of time spent scratching one's self and rubbing on pen fixtures.

### 6.3.4 Sample Analysis

Herbage samples were thoroughly mixed and subsampled into three parts, to determine botanical composition, DM, and herbage chemical composition. The subsample of herbage taken to determine DM was weighed, dried at 60°C for 7 days, and re-weighed dry. Botanical and chemical composition samples were analyzed every four days. The botanical sub-samples of the herbage were also dried at 60°C for 7 days after sorting into leaf, stem, weeds, and dead material. The chemical composition of freeze-dried and ground (ZM200; Retsch, Haan, Germany; 1mm screen) herbage samples were determined using near-infrared spectrophotometry (NIRS; Model: FOSS NIRS Systems 5000, Maryland, USA). Chemical composition values used for NIRS calibration were derived before sample analysis for DM (AOAC International, 1990; method 930.15), organic matter (OM; 100% minus ash%; AOAC International, 1990; method 942.05), neutral detergent fiber (NDF; Van Soest et al., 1991), acid detergent fiber (ADF; AOAC International, 1990; method 973.18), water-soluble carbohydrates (WSC; MAFF, 1986), digestible OM in DM (DOMD), DM digestibility (DMD), and OM digestibility (OMD; lowerth et al., 1975), and crude protein (CP) by combustion (Variomax CN Analyser; Elementar Analysensysteme, Hanau, Germany). The NIRS calibration equations all had R<sup>2</sup> values greater than 0.90 and were within the calibration range. Herbage metabolizable energy (ME) was estimated using the Primary Industries Standing Committee (2007) equation:

$$[\text{ME (MJ/kg DM)} = \text{digestible OM in DM, \% (DOMD)} \times 0.16] \quad [1].$$

The GPx content of the whole blood samples was determined using an enzymatic-based protocol (RANSEL; Cat. No. RS504) and a clinical analyzer (Randox Rx Daytona, Crumlin, Co. Antrim, UK). The TAS content of the plasma was determined using a colorimetric method on the clinical analyzer using a commercial kit (Cat. No. NX2332; Randox Rx Daytona, Crumlin, Co. Antrim, UK).

Ammonia concentration of the acidified rumen samples was measured using a clinical analyzer (Randox Rx Daytona, Crumlin, Co. Antrim, UK) and a commercial test kit (Cat. No. AM3979; Randox; Crumlin, Co. Antrim, UK) based on the enzymatic UV method described by Neeley and Phillipson (1988). Rumen volatile fatty acid (VFA) concentration of the non-acidified samples was determined using a Gas Chromatograph (GC; Shimadzu GC-2010, Kyoto, Japan with AOC-20i auto-sampler) fitted with a SGE BP21 30 m × 530 μm × 1 μm wide-bore capillary column as described by Chen and Lifschitz (1989). The lactate concentration of the non-acidified rumen fluid was determined using a separate commercial kit (Cat. No. LC2389; Randox; Crumlin, Co. Antrim, UK) and the Randox Rx Daytona clinical analyzer.

### 6.3.5 Statistical Analysis

Statistical analysis was conducted using R (R Core Team, 2018, v.3.6.0). All normally distributed data ( $P > 0.10$ ; Shapiro-Wilk test) that had homogenous variance ( $P > 0.10$ ; Bartlett's test) were analyzed using an analysis of variance (ANOVA) using the 'aov' function. Data analyzed using 'aov' function included: DMI, ADG, and FCE. Non-normally distributed data were analyzed by a generalized linear model (GLM) using the 'glm' function of R (R Core Team, 2018) with the distribution used for the model selected based on qq-plots of the residuals. Non-normally distributed data included: rumen ammonia, rumen glucogenic VFA, rumen non-glucogenic VFA, total VFA. Differences in chemical composition among herbage and diets were tested using the 'lme' mixed model function, with day as a random effect. The d1 samples (pre-treatment) for the rumen variables were explored as covariates and included in the model as they explained a significant ( $P < 0.05$ ) amount of the variation in rumen parameters. The ANOVA and GLM models that contained repeated measures (i.e. blood and rumen variables) included diet, time, and the diet  $\times$  time interaction as fixed effects. A random effect was included to account for the repeated measures of blood and rumen. The models for variables of averaged data or that were not repeatedly measured (i.e. DMI, ADG, and FCE) contained diet as fixed effects. The behavior data model included the treatment, observation time (morning = dawn to noon; afternoon = noon to dusk), observation days, and their interactions as fixed effects. Upon significance of the ANOVA, means separation among treatments was conducted by a pairwise t-test using the 'emmeans' package (Lenth, 2018). The DMI data were used to calculate the within animal day-to-day coefficient of variation (CV) of DMI. Pearson's correlation coefficient between the day-to-day variability in DMI (CV) and DMI, ADG, and FCE was determined using the 'cor.test' function of R (R Core Team, 2018). Statistical significance was declared at  $P \leq 0.05$  with tendencies declared at  $0.05 < P \leq 0.10$ .

## 6.4 Results

### 6.4.1 Diet Composition

The DM of the alfalfa and ryegrass herbage were not different to one another ( $P > 0.05$ ), however their DM was 72% greater ( $P < 0.05$ ) than chicory and plantain, which were not different from one another ( $P > 0.05$ ; Table 6.1). While the ME of alfalfa and ryegrass were not different ( $P > 0.05$ ), they were 10% lower than chicory and plantain ( $P < 0.05$ ), which were not different ( $P > 0.05$ ). Chicory and plantain had the least ( $P > 0.05$ ) CP, ryegrass was intermediate ( $P < 0.05$ ), and alfalfa had the greatest CP content ( $P < 0.05$ ). The chemical compositions of each diet consumed are reported in Table 6.2. There were no differences ( $P > 0.05$ ) in the chemical composition (e.g. ME, DM, DMD, OM, OMD, WSC, and CP) between the DIV and VAR diets. There were no treatment differences for NDF contents of the diets consumed ( $P = 0.22$ ); however, there was a tendency ( $P = 0.07$ ) for the SFA diet

to have a greater ADF content than the DIV and VAR diets. The ME and WSC content of the DIV and VAR diets were greater ( $P < 0.05$ ) than the SFA diet. Conversely, the CP content of the SFA diet was greater ( $P < 0.05$ ) than both the DIV and VAR diets. Leaf comprised 87.4, 77.6, 96.1, and  $81.4 \pm 2.9$  % of the DM respectively for chicory, plantain, alfalfa, and ryegrass, respectively. The DM comprised of weed for chicory, plantain, alfalfa, and ryegrass was 11.3, 0.9, 3.7, and  $0.8 \pm 2.9$  % respectively. While, dead matter made up 1.3, 2.5, 0.2, and  $10.9 \pm 2.9$  % for chicory, plantain, alfalfa, and ryegrass respectively. Only plantain and ryegrass had any stem material, with 19.0 and  $6.9 \pm 3.1$  % each.

#### **6.4.2 Forage DMI and ADG**

The DIV lambs consumed 6 % more ( $P = 0.01$ ) total DM compared with SFA lambs, while the VAR lambs were intermediate and not different ( $P = 0.15$ ) compared to the other treatments (Table 6.4). Although the DMI of the DIV and VAR treatments were not different the proportions of species they consumed to reach that level of intake differed. The SFA treatment consumed 163% more ( $P < 0.01$ ) alfalfa on a DM –basis compared to the DIV or VAR lambs, which did not differ ( $P > 0.05$ ) in alfalfa intake. The VAR lambs had a 14% greater chicory intake ( $P < 0.05$ ), but 26 % less plantain intake ( $P < 0.05$ ) compared with the DIV lambs. There was a tendency for the VAR lambs to consume more DM as ryegrass than the DIV lambs ( $P < 0.10$ ). The within animal between days DMI CV was 30% greater for the SFA treatment compared with the VAR treatment ( $P < 0.05$ ). There was a tendency for the DIV lambs to have a lower within animal between day DMI CV compared to the SFA lambs ( $P = 0.08$ ); however, there was no difference ( $P > 0.05$ ) among the DIV and VAR lambs ( $P > 0.05$ ). The ADG of DIV lambs (296 g/d) was 30% greater ( $P < 0.05$ ) compared with SFA (227 g/d). The ADG for VAR lambs (378 g/d) was 28 and 67% greater ( $P < 0.05$ ) than the DIV and SFA lambs, respectively. The FCE of VAR was 63% greater ( $P < 0.01$ ) than the SFA lambs and 30% greater ( $P < 0.05$ ) than the DIV lambs. The DIV lambs tended ( $P < 0.10$ ) to have a 25% greater FCE compared with the SFA lambs.

#### **6.4.3 Rumen and Blood**

Rumen ammonia ( $\text{NH}_3$ ) concentration at the morning sampling (0800 h) was 287% greater for the SFA lambs compared with the VAR lambs, while DIV was intermediate and different ( $P < 0.05$ ) to SFA and VAR (Table 6.5). At the afternoon sampling (1700 h) rumen  $\text{NH}_3$  concentrations of VAR and DIV were not different ( $P > 0.05$ ), but they were both lower ( $P < 0.05$ ) than the SFA lambs. While the rumen  $\text{NH}_3$  concentrations did not differ ( $P > 0.05$ ) between the morning and afternoon for the SFA and VAR, the rumen  $\text{NH}_3$  of the DIV treatment was lower ( $P < 0.05$ ) in the afternoon compared to the morning.

There was no interaction between time of day and treatment ( $P = 0.50$ ) and there was no overall treatment effect ( $P = 0.13$ ) on total VFA concentration, but there was a time of day effect ( $P = 0.02$ ), with total VFA concentration being greater in the afternoon. A time of day  $\times$  treatment interaction ( $P = 0.03$ ) was detected for the acetate to propionate ratio, with VAR having lower ( $P < 0.05$ ) acetate to propionate ratio than the DIV and SFA in the morning, but no effect was detected ( $P > 0.05$ ) in the afternoon. There was a tendency ( $P = 0.07$ ) for a time of day  $\times$  treatment interaction for the percentage of VFA that were glucogenic and non-glucogenic, with the VAR treatment having a greater percentage of glucogenic VFA in the morning.

Time of day  $\times$  treatment interaction for plasma TAS ( $P < 0.01$ ) was detected (Fig.1). No difference was detected among treatments at 0 ( $P > 0.05$ ); however, at 6 h the VAR treatment TAS ( $1.36 \pm 0.02$  mmol/L) was lower than the DIV ( $1.44 \pm 0.03$  mmol/L;  $P < 0.01$ ) and SFA ( $1.43 \pm 0.02$  mmol/L;  $P = 0.02$ ), which were not different to one another ( $P = 0.69$ ), and at 12 h the VAR treatment ( $1.60 \pm 0.03$  mmol/L) had a TAS concentration that was 11% greater than the SFA ( $1.44 \pm 0.03$  mmol/L;  $P < 0.01$ ). At 12 h the DIV lambs' TAS concentration ( $1.56 \pm 0.03$  mmol/L) was 8% greater than the SFA ( $P = 0.03$ ) but not different to the VAR treatment ( $P = 0.56$ ). The TAS concentration of all treatments was greater at the 12 h measurement compared to the 0 hr measurement ( $P < 0.05$ ). There was only a time of day effect on GPx concentration ( $P < 0.05$ ), with GPx concentrations being greater earlier in the day.

#### **6.4.4 Behavioral Observations**

There was a treatment ( $P < 0.01$ ; Table 6.6) and time effect ( $P < 0.01$ ) on the proportion of time spent eating in the morning and afternoon, and a treatment effect ( $P < 0.01$ ) over the whole day. For each time of day and over the whole day the proportion of time eating was greatest for the SFA lambs, intermediate for DIV lambs, and lowest for the VAR lambs, all of which were different ( $P < 0.05$ ) from one another. Similarly, there was a treatment effect ( $P = 0.03$ ) on the proportion of time spent ruminating in the morning, afternoon, and over the whole day. In addition, there was a time effect ( $P < 0.01$ ), but no treatment  $\times$  time interaction ( $P = 0.53$ ) on the proportion of time spent ruminating in the morning and afternoon. During the morning and afternoon, and over the whole day the VAR lambs spent more time ( $P < 0.05$ ) ruminating than both the SFA and DIV treatments, which were not different ( $P > 0.05$ ). There was a treatment ( $P < 0.01$ ) effect on the proportion of time lambs spent idle in the morning and afternoon, and over the whole day, with the SFA treatment spending less time idle than the DIV and VAR treatments, which were not different ( $P > 0.05$ ).

There was a treatment ( $P < 0.01$ ) effect on the proportion of time spent lying down in the morning, afternoon, and over the whole day. In addition, there was a time ( $P < 0.01$ ) effect on the proportion of time spent lying down in the morning and afternoon. At each of the time periods and

over the whole day, the time spent lying was not different ( $P > 0.05$ ) for the VAR and DIV treatment; however, they spent more time ( $P < 0.05$ ) lying than the SFA treatment. Conversely, the SFA lambs spent a greater ( $P < 0.05$ ) proportion of time standing at each time point analyzed over the day compared with the DIV and VAR lambs, which did not differ ( $P > 0.05$ ) from one another.

There was a treatment  $\times$  time interaction ( $P < 0.01$ ) on the number of stereotypic behavior bouts recorded in the morning and afternoon. Over the whole day, the SFA lambs had a greater ( $P < 0.05$ ) number of stereotypic behavior bouts than the DIV and VAR lambs, which did not differ ( $P > 0.05$ ). In the morning all treatments had a different number of stereotypic behavior bouts ( $P < 0.05$ ), the SFA treatment had the greatest number bouts, followed by the DIV, and then VAR. In the afternoon, the SFA lambs had 81% more bouts ( $P < 0.05$ ) of stereotypic behaviors than the DIV lambs. During this afternoon period, the incidence of stereotypic behavior bouts by VAR treatment was intermediate and not different ( $P > 0.05$ ) to the SFA and DIV treatments. There was a treatment ( $P < 0.01$ ) and time ( $P < 0.01$ ) effect on the number of bouts of grooming recorded. In addition, over the whole day there was a treatment effect ( $P < 0.05$ ). At each of the periods examined, the VAR treatment had a greater ( $P < 0.05$ ) number of grooming bouts compared to the SFA and DIV treatments, which were not different ( $P > 0.05$ ) to one another.

## **6.5 Discussion**

We hypothesized that a diverse and varied diet of fresh forages over the day will improve intake, performance, and welfare of lambs compared to dietary monotony and that the varied diet of fresh forages over the day would improve intake, performance, and welfare of lambs compared to dietary diversity of the same herbage species on offer at the same time. Based on the results we accept that diverse and varied diets can improve performance and welfare relative to a single forage monotony, however reject that a varied diet will improve intake within this context. We also accept that a varied diet can improve performance compared with a diverse diet. The following sections will discuss the intake and performance of the SFA treatment compared to the DIV and VAR and then compare the DIV and VAR treatments. The rumen, blood, and behavior data of the treatments are then discussed collectively to give inferences on potential welfare differences. Finally, we outline areas for future research as identified by this work.

### **6.5.1 Intake and Performance: SFA vs. DIV and VAR**

A diverse diet that is varied through time can improve performance relative to alfalfa or a free choice diverse diet that is presented in a monotonous manner. The DIV and VAR lambs both had greater ADG than the SFA, largely explained by differences in the nutritional composition of the diet, namely the greater DMD and ME content of the DIV and VAR diets. Other studies have reported

similar results with lower DMI from treatments offered a flavourally or biochemically uniform diet compared to those offered choice from a diverse range of feeds (Keskin et al., 2004; Atwood et al., 2006; Distel et al., 2007; Villalba et al., 2011; Garrett et al., 2021). The magnitude of the effect from choice of diverse feeds on increased DMI is lower within the present study than that reported in other studies. For example, Garrett et al. (2021) reported a 48% increase in DMI from lambs of the same age and similar weights offered choice from a set ratio of chicory, alfalfa, plantain, and ryegrass compared with those repetitively fed ryegrass. Discrepancies between Garrett et al. (2021) and the current study may be a result of the different forage species (i.e., ryegrass vs. alfalfa) and their chemical composition. Greater DMI by lambs and other ruminants grazing alfalfa have been reported compared to those grazing ryegrass, even when the *in vivo* digestibility was similar (Niezen et al., 1993; Fraser et al., 2004). Greater DMI of alfalfa diets compared to ryegrass diets is a result of legume forages being more susceptible to ingestive and digestive particle breakdown, increasing rumen clearance rate, and thereby reducing the physical constraint of intake (Waghorn et al., 1989; Jamot and Grenet, 1991; Mertens, 1994). Greater ingestive and digestive particle breakdown of alfalfa may also explain why, despite having a greater predicted digestibility (+7.5%), the DIV and VAR did not have greater intakes, respectively, than the SFA diet. Varying the availability of diverse diet components throughout the day can reduce the DMI CV compared to repeatedly allocating alfalfa. The greater DMI CV of the SFA treatment suggest that the lambs within the treatment were forming short-term aversions to alfalfa, thus creating cyclic patterns of intake (Provenza, 1996). Improved performance (i.e. ADG) with reduced CV has been reported by several studies (Allison, 1985; Galyean et al., 1992; Horn et al., 2005; Williams et al., 2018), which allow us to suggest that the greater ADG of DIV and VAR could have been due to more consistent feed consumption as well as differences in the primary composition of diets, compared with the SFA. Further, choice of diet components as available to the DIV and VAR treatments has been shown to allow animals to better fulfil their nutrient requirements, thereby increasing the feed conversion efficiency of the consumed herbage (Atwood et al., 2001). For example, Atwood et al. (2001) offered calves free-choice from a diet components comprising a total mixed ration (TMR) or TMR and found that on average both treatments consumed a diet of similar energy to protein ratios, however the results suggested individual intake of diet components varied greatly within the free-choice group suggesting that the no-choice TMR group were potentially over-ingesting energy to meet their protein needs.

Utilizing first principles based on energy requirements for maintenance and growth and the measured diet nutritive value, an estimated ADG of 274, 354, and 349 g/d for the SFA, DIV, and VAR treatments was calculated, respectively (Nicol and Brookes, 2007). These calculations predict similar ADG for the DIV and VAR treatments (354 vs 349 predicted g/d, respectively), which were not similar to the recorded ADG (296 vs 378 g/d) for the DIV and VAR treatments. We argue that such a model

only accounts for the intake of primary nutrients and we now know that PSC can impact animals at a range of levels including their intake and performance. For example, alfalfa is known to be a rich source of plant secondary compounds, particularly saponins, but also flavonoids and phenolics (Rafińska et al., 2017). Saponins from alfalfa are known to reduce microbial fermentation, protozoa numbers, and the digestion of nutrients, and have been suggested to adversely affect rumen microbial protein production (Lu and Jorgensen, 1987). Thereby as animal production (growth, development, and reproduction) is greatly influenced by nutrient utilization it is reasonable to assume that these anti-nutritional properties of alfalfa may contribute to the lower feed conversion efficiency and production of the SFA diet.

The single forage diet had a greater rumen  $\text{NH}_3$  concentration compared with the VAR and DIV treatments, whom could reduce their rumen  $\text{NH}_3$  concentration through dilution of protein intake from alfalfa, thereby balancing the soluble protein to energy ratio (Hill et al., 2009). As a result of the excessive dietary nitrogen (evidenced by the elevated rumen  $\text{NH}_3$  concentration) it is likely that the SFA animals experienced the resulting negative post-ingestive effects to a greater degree than the other treatments. Elevated rumen  $\text{NH}_3$  is associated with increased blood  $\text{NH}_3$ , both of which represent a toxin burden to the animal which requires negation through assimilation into amino acids or excretion via urine (Hill et al., 2009). Plant primary and secondary components act in a nutrient to toxin concentration gradient, where the actions of ingested compounds have dose dependent effects (Raubenheimer et al., 2009; Beck and Gregorini, 2020). The finding of elevated  $\text{NH}_3$  are similar to the findings of Dziba and Provenza (2007) and Dziba et al. (2006) who found that intake of high levels of monoterpenes (a PSC), resulted in negative post-ingestive feedback and induced satiety. The DIV and VAR diets would have ingested a greater range of different kinds and quantities PSC compared to the SFA, which would be detoxified through different mechanisms at different rates (Freeland and Janzen, 1974), thereby reducing the chance of a detoxifying pathway being saturated and thus reducing any associated negative effects. For example, the DIV and VAR treatments had access to chicory which is rich in tannins, flavonoids, coumarins, sesquiterpene lactones, and alkaloids (Nwafor et al., 2017), plantain rich in iridoid glycosides, aucubin and catapol, and tannins (Rumball et al., 1997), and perennial ryegrass is rich in endophyte alkaloids, flavonoids, and phenolics (Cao et al., 2017; Kagan, 2021). Therefore, perhaps diverse and varied diets providing a range of primary and secondary compounds can negate some of the nutritional inefficiencies or toxic effects encountered when a monotonous diet is supplied. For example, each the DIV and VAR diets consumed chicory which contains tannins that form complexes with proteins, altering protein digestion and aiding in alleviated the adverse effects of too much protein in the diet (Naumann et al., 2017).

### 6.5.2 Intake and Performance: DIV vs VAR

In addition to greater capability of avoiding or negating toxicosis animals offered a diverse diet are also thought to have greater capability of ingesting an appropriate dose of compounds that allows for increased utilization of beneficial therapeutic properties (Provenza et al., 2007; Dixon and Pasinetti, 2010), and have even been suggested to increase the efficiency of rumen fermentation (Frutos et al., 2008). Although the plant primary chemical composition of the VAR and DIV diets were not different, the proportions of species consumed and thereby the quantities of ingested PSC to reach this composition likely differed and contributed to the differences in performance seen. The ingestion of PSC from different plants can have synergistic effects, offering greater benefits than what an individual plant species can offer (Tilman, 1982; Gregorini et al., 2017). Thereby ingestion of a different quantity of a particular PSC or a different ratio of PSC may have resulted in greater exploitation of a property or synergistic effect that increased efficiency of the VAR treatment in comparison with the DIV treatment. For example, the

Differences in ruminal parameters between the DIV and VAR provide evidence for differences in nutrient use efficiency between the VAR and DIV lambs. For example, the VAR treatment had a lower acetate:propionate ratio compared with the DIV in the AM, which is indicative of increased energy retention by the animal (Russel, 1998; Wolin, 1960). Further, in the AM the DIV had a greater rumen  $\text{NH}_3$  compared with the VAR treatment. Elevated  $\text{NH}_3$  can indicate inefficiencies as the nitrogen availability exceeds the capacity for microbial utilization (Chanu et al., 2020). The VAR treatment consumed its DM through small quantities of ryegrass and plantain in the morning. Plantain, which has a lower CP concentration, reduces production of rumen  $\text{NH}_3$  through the presence of aucubins and acetocides (Navarrete et al., 2016; Nkomboni, 2017), when it is increased at a level of 30% or greater as it is in the AM by the VAR. In the PM when the VAR treatment consumed most of its protein through alfalfa, there was no rise in  $\text{NH}_3$  levels, indicating protein was effectively utilized. It is possible that the greater intake of chicory by the VAR animals, aided in this effect, with the chicory tannins binding some of the protein, reducing the amount of rumen degradable protein and increasing the portion of non-ammonia N reaching the small intestine and thereby the ratio of essential amino acids to energy (Villalbla et al., 2015; Waghorn et al., 1987). However, the DIV treatment paired a greater number of feeds during any feeding period, perhaps this resulted in the complexation of PSC to negate any increases in nutrient use inefficiencies. Further, the DIV treatment had a lower intake of chicory, thereby would likely have had a corresponding decrease in the quantity of tannins ingested and the beneficial properties associated with this. At the same level of intake with no differences in dietary CP, the VAR treatment had lower rumen  $\text{NH}_3$  levels in the AM and no difference in the PM than the DIV treatment, indicating reduced release of ammonia from soluble protein.



The results indicate that diverse and varied diets can improve production further than a currently common and high performing feed (e.g. SFA). Although, future testing of the PSC profiles of herbage offered is required, and a control diet containing a homogenized un-sortable mixture of the diverse plants as a dietary control or offering a monotonous diet of all single forages comprising the diverse diet could allow for treatment comparisons of more similar PSC profiles. Another contributing cause of comparatively lower performance from the DIV treatment relative to the VAR treatment, despite similar DMI and primary chemical composition of diets, could be that the DIV treatment experienced a greater level of stress, potentially indicated by the differences in stereotypic behavior in the AM. For example, elevated levels of glucocorticoids in the blood of stressed animals elicit physiological responses that result in reduced feed conversion efficiency (Llonch et al., 2016). This premise may explain why the FCE (g ADG/kg DMI) was not different between the DIV and SFA treatments but was greater for the VAR treatment.

### **6.5.3 Rumen, Blood, and Behavior Indicating Differences in Welfare: SFA vs. DIV vs. VAR**

Greater animal performance (Roche et al., 2009; Barrell, 2019) and more consistent DMI (McGuffey et al., 1997) have been associated with reduced health incidents and improved welfare. Thereby the order of increasing ADG; SFA < DIV < VAR may also be indicative of the hierarchy of welfare among the treatments. In addition, excessive levels of dietary N can have detrimental effects on animal health and thereby welfare (Pacheco and Waghorn, 2008). Thus, the elevated concentration of rumen NH<sub>3</sub> of the SFA relative to the DIV and VAR may be suggestive of reduced welfare. The VAR treatment had a greater number of grooming bouts compared with the DIV and SFA treatments, which can be considered an indicator of positive welfare (Napolitano et al., 2009). Further, the VAR and DIV treatments exhibited fewer bouts of stereotypic behaviors than the SFA treatment. Although stereotypic behaviors are only partial indications of impaired welfare (Mason, 1991), we argue, as per Garrett et al., (2021), that such behaviors should be minimized where possible to enhance animal wellbeing.

Varying allocation of diverse diet components can alter diurnal patterns of DMI, which is perhaps the cause of differences in TAS levels during subsequent measurements. Dietary antioxidants (e.g. vitamin E and PSC) are a major exogenous defense against oxidative damage. As such, greater antioxidant status can be a sign of improved internal state and well-being of ruminants (Beck and Gregorini, 2020). At 15:30 h the VAR treatment had a lower TAS than either DIV or SFA, likely a result of consuming very little feed containing antioxidants prior to this measurement. We do not believe that the lowered TAS level at this time is indicative of elevated stress due to the VAR treatment also exhibiting fewer bouts of stereotypic behaviors than both other treatments over this period. At 21:30 h the VAR and DIV treatments had a greater TAS than the SFA treatment, perhaps

because their diet likely containing greater levels of antioxidants, in the period leading up to this sampling. Alternatively, the SFA may have experienced a greater level of oxidative stress over the day, supported by their greater levels of stereotypic behavior throughout the day, depleting their TAS levels, relative to the DIV and VAR treatments.

Overnight grazing activity is typically reduced as it diminishes alertness and thereby increases the risk of predation (Gregorini, 2012). Thereby, based on the elevated TAS levels at nightfall for the VAR and DIV animals, we speculate that these treatments are better prepared to cope with stressors encountered overnight, which is considered a stressful time as animals are more susceptible to predation at night (Tyler et al., 2016). We therefore argue that, by offering a diverse or varied diet, farmers can enhance animal welfare by increasing the antioxidant levels available to combat oxidative stress or perhaps aid in preventing stress that would otherwise deplete antioxidants overnight.

Collectively, our results — reduced DMI CV, increased ADG and 12 h TAS, reduced bouts of stereotypic behavior, and increased grooming (VAR only)— present compelling evidence in support of the DIV and VAR lambs having increased welfare relative to the SFA lambs. Further, the increased ADG and grooming bout number of the VAR treatment compared to the DIV may be indicative of improved welfare but further research with more definitive measures of welfare are required e.g. cortisol in blood, saliva, feces, or wool.

#### **6.5.4 Future Research**

Our research is some of the first to depict that varying forage availability through time can improve performance of lambs relative to those offered continuous access to the same diverse forages. Although our research was some of the first to depict such a phenomenon, future research repeating such experimentation with greater animal numbers would further strengthen the results seen. Due to limitations of resources the current experiment was only able to examine the effect of one feeding sequence, future research exploring different forage combinations and the possible sequences could elucidate patterns of forage offerings that could enhance performance. Future examination of sequence effects should be done in grazing situations to evaluate and examine how diurnal fluctuations in forage composition impact the results reported here. Further research is also required on the duration at which varied diet sequences still elicit an effect, for example would one week on ryegrass and plantain and then another week on chicory an alfalfa still elicit the benefits seen or is the time scale used within the present study important.

## 6.6 Conclusions

The diverse and varied diets explored improve animal performance relative to high performance diet of alfalfa fed monotonously. Further, temporal management of diverse diets (to create varied diets e.g. VAR) can improve performance relative to animals given free choice diversity at all times. Moreover, the diverse and varied diets may enhance animal welfare in comparison with a monotonous alfalfa diet, and a varied diet may provide welfare advantages to the repetitive presentation of free choice diversity. Although, the exact mechanism for this increased performance of the varied diet compared with the diverse is unclear within the present study, therefore requiring further evaluation, it highlights that it is more than merely the primary chemical composition of the diet consumed but rather how the diet is presented through time, and the herbage species and quantities of each species consumed to reach that primary composition that influences performance and animal welfare.

**Table 6.1** Chemical composition of the herbage eaten composing the total diets.

Item <sup>2</sup>	Herbage				SEM <sup>1</sup>
	Chicory	Alfalfa	Plantain	Ryegrass	
DM, % as-is	13.34 <sup>b</sup>	21.67 <sup>a</sup>	12.56 <sup>b</sup>	22.86 <sup>a</sup>	0.63
OM, %DM	86.80 <sup>c</sup>	91.00 <sup>b</sup>	91.72 <sup>ab</sup>	92.01 <sup>a</sup>	0.36
CP, % DM	12.27 <sup>c</sup>	21.11 <sup>a</sup>	13.39 <sup>c</sup>	16.17 <sup>b</sup>	0.60
NDF, % DM	16.85 <sup>c</sup>	25.46 <sup>b</sup>	24.54 <sup>b</sup>	49.91 <sup>a</sup>	1.17
ADF, % DM	18.59 <sup>c</sup>	23.27 <sup>b</sup>	21.90 <sup>b</sup>	30.94 <sup>a</sup>	0.79
WSC, % DM	18.17 <sup>b</sup>	11.81 <sup>c</sup>	29.40 <sup>a</sup>	16.71 <sup>b</sup>	0.98
DMD, %DM	83.86 <sup>a</sup>	72.98 <sup>bc</sup>	75.00 <sup>b</sup>	71.46 <sup>c</sup>	1.16
OMD, % OM	88.61 <sup>a</sup>	76.62 <sup>c</sup>	81.39 <sup>b</sup>	76.47 <sup>c</sup>	1.27
ME, MJ/kg DM	12.80 <sup>a</sup>	11.13 <sup>b</sup>	12.53 <sup>a</sup>	11.70 <sup>b</sup>	0.26

<sup>a-d</sup> Means in a row with different superscripts are statistically different ( $P \leq 0.05$ ).

<sup>1</sup> SEM = standard error of the mean.

<sup>2</sup> DM = dry matter; OM = organic matter; CP = crude protein; NDF = neutral detergent fiber; ADF = acid detergent fiber; WSC = water soluble carbohydrates; DMD = dry matter digestibility; OMD = OM digestibility; ME = metabolizable energy

**Table 6.2** Chemical composition of the single forage alfalfa (SFA) diet and the calculated chemical composition of the diverse (DIV) and varied (VAR) diet consumed by the ram lambs.

Item <sup>1</sup>	Treatment Diet <sup>2</sup>			SEM <sup>3</sup>	P <sup>4</sup>
	SFA	DIV	VAR		
DM, % as-fed	21.67 <sup>a</sup>	16.82 <sup>b</sup>	16.75 <sup>b</sup>	0.76	<0.01
OM, % DM	91.33 <sup>a</sup>	90.10 <sup>b</sup>	90.29 <sup>b</sup>	0.39	0.03
CP, % DM	20.49 <sup>a</sup>	17.17 <sup>b</sup>	16.41 <sup>b</sup>	0.68	<0.01
NDF, % DM	28.27	25.18	26.63	1.49	0.22
ADF, % DM	25.08	22.89	22.88	0.58	0.07
WSC, % DM	11.29 <sup>b</sup>	17.33 <sup>a</sup>	18.36 <sup>a</sup>	1.11	<0.01
DMD, % DM	70.87 <sup>b</sup>	75.94 <sup>a</sup>	76.40 <sup>a</sup>	1.17	<0.01
OMD, % DM	74.08 <sup>b</sup>	81.08 <sup>a</sup>	81.03 <sup>a</sup>	0.90	<0.01
ME, MJ/kg DM	10.78 <sup>b</sup>	11.94 <sup>a</sup>	12.08 <sup>a</sup>	0.16	<0.01

<sup>a-c</sup> Means in a row with different superscripts are statistically different ( $P \leq 0.05$ ).

<sup>1</sup> ME = metabolizable energy; DM = dry matter; OM = organic matter; OMD = OM digestibility; WSC = water soluble carbohydrates; NDF = neutral detergent fiber; ADF = acid detergent fiber; CP = crude protein.

<sup>2</sup> Values for diverse diet chemical composition were calculated by using the percentage of the Item value that each dietary component accounted for. SFA = monotonous alfalfa diet; DIV = free choice of diverse diet components: chicory, ryegrass, plantain, and alfalfa; VAR = free choice of plantain and ryegrass in the morning and chicory and alfalfa in the afternoon.

<sup>3</sup> SEM = Standard error of the mean.

<sup>4</sup> P = t-test P-value.

**Table 6.3** Ethogram of recorded behavioral activities and the category they were considered under.

Behavior	Description
Eating	Eating specific was recorded
Idle	Sheep not engaged in any of the other listed behaviors
Ruminating	Sheep is ruminating
<b>Position</b>	
Standing	Sheep is in an upright position
Lying	Sheep is lying down
<b>Stereotypic behaviors</b>	
Pacing	Walking in a distinct pattern, such as frequent walking back and forth, weaving, or moving in circles
Chewing pen fixtures	Chewing pen fixtures (e.g. feed bin, bars)
Head butting pen fixtures	Butting pen fixtures
Head hanging	Standing quietly with head drooped down
Crouching	Crouching in fear (usually to human activity)
Pawing or stamping	Striking ground with forelegs
Rearing	Head raised with forelegs on pen or off ground, back legs on ground
<b>Grooming behaviors</b>	
Scratching	Scratching self
Rubbing	Rubbing on pen fixtures

**Table 6.4** Mean dry matter intake, growth, and feed conversion efficiency of ram lambs fed a varied (VAR), diverse (DIV), and single forage alfalfa (SFA) diet over 20 days

Item <sup>1</sup>	Treatment <sup>2</sup>			SEM <sup>3</sup>	P <sup>4</sup>
	SFA	DIV	VAR		
Initial LW, kg	33.9	33.9	32.8	1.0	0.63
Total DMI, kg DM/ d	1.54 <sup>b</sup>	1.64 <sup>a</sup>	1.59 <sup>ab</sup>	0.03	0.04
AM	0.70 <sup>a</sup>	0.70 <sup>a</sup>	0.41 <sup>b</sup>	0.02	<0.01
PM	0.84 <sup>c</sup>	0.94 <sup>b</sup>	1.18 <sup>a</sup>	0.03	<0.01
Alfalfa DMI, kg DM/ d	1.54 <sup>a</sup>	0.62 <sup>b</sup>	0.55 <sup>b</sup>	0.03	<0.01
AM	0.70 <sup>a</sup>	0.30 <sup>b</sup>	—	0.01	<0.01
PM	0.84 <sup>a</sup>	0.32 <sup>c</sup>	0.55 <sup>b</sup>	0.02	<0.01
Chicory DMI, kg DM/ d	—	0.58 <sup>b</sup>	0.66 <sup>a</sup>	0.02	<0.01
AM	—	0.23	—	0.01	<0.01
PM	—	0.35 <sup>b</sup>	0.66 <sup>a</sup>	0.01	<0.01
Plantain DMI, kg DM/ d	—	0.31 <sup>a</sup>	0.23 <sup>b</sup>	0.01	<0.01
AM	—	0.12 <sup>b</sup>	0.23 <sup>a</sup>	0.01	<0.01
PM	—	0.19	—	0.01	<0.01
Ryegrass DMI, kg DM/ d	—	0.13	0.15	0.01	0.08
AM	—	0.05 <sup>b</sup>	0.15 <sup>a</sup>	0.01	<0.01
PM	—	0.08	—	0.01	<0.01
DMI CV, %	22.34	17.44	17.13	2.35	0.10
ADG, g BW/d	227 <sup>c</sup>	296 <sup>b</sup>	378 <sup>a</sup>	22	<0.01
FCE, g BWgain/ kg DMI	146 <sup>b</sup>	183 <sup>b</sup>	238 <sup>a</sup>	14	<0.01

<sup>a-c</sup> Means in a row with different superscripts are statistically different ( $P \leq 0.05$ ).

<sup>1</sup> Initial LW = initial live weight; DMI = dry matter intake; DMI CV = day-to-day DMI co-efficient of variation; ADG = average daily gain; FCE = feed conversion efficiency; AM = 0700 h to 1600 h; PM = 1600 h to 0700 h.

<sup>2</sup> SFA = Single forage diet of alfalfa; DIV = free choice of diverse diet components: chicory, ryegrass, plantain, and alfalfa; VAR = free choice of plantain and ryegrass from 0700 – 1600 h and chicory and alfalfa between 1600 – 0700 h.

<sup>3</sup> SEM = Standard error of the mean.

<sup>4</sup> P = t-test P-value.

**Table 6.5** Rumen ammonia (NH<sub>3</sub>) and rumen volatile fatty acid (VFA) profile of ram lambs on d17 in the morning and afternoon.

Item <sup>1</sup>	Treatments <sup>2</sup>						P-value <sup>3</sup>		
	SFA		DIV		VAR		Time	TRT	Time×TRT
	AM	PM	AM	PM	AM	PM			
NH <sub>3</sub> , mmol/L	16.13 <sup>a</sup> ±1.06	14.04 <sup>a</sup> ±1.06	8.72 <sup>b</sup> ±1.11	5.57 <sup>c</sup> ±1.11	4.16 <sup>c</sup> ±1.06	5.28 <sup>c</sup> ±1.03	0.14	<0.01	0.11
Total VFA, mmol/L	139 <sup>ab</sup> ±9	145 <sup>a</sup> ±9	113 <sup>bc</sup> ±10	141 <sup>a</sup> ±10	112 <sup>c</sup> ±10	134 <sup>abc</sup> ±10	0.02	0.13	0.50
Ace:Prop, ratio	3.06 <sup>a</sup> ±0.16	2.90 <sup>a</sup> ±0.16	2.88 <sup>a</sup> ±0.17	2.67 <sup>ab</sup> ±0.17	2.31 <sup>b</sup> ±0.16	2.86 <sup>a</sup> ±0.16	0.34	0.04	0.03
VFA profile, %									
Glucogenic	23.98±1.06	24.75±1.06	24.87±1.16	26.38±1.16	27.57±1.07	24.40±1.07	0.66	0.28	0.07
Nonglucogenic	76.02±1.06	75.25±1.06	75.13±1.16	73.62±1.16	72.43±1.07	75.60±1.07			

<sup>a-c</sup> Means in a row with different superscripts are statistically different ( $P \leq 0.05$ ).

<sup>1</sup>NH<sub>3</sub> = ammonia, mmol/L; Total VFA = total volatile fatty acid (mmol/L); Ace:Prop ratio = ratio of acetate to propionate; Gluc. = glucogenic VFAs; Non. = non-glucogenic VFAs.

<sup>2</sup> SFA = single forage diet of alfalfa; DIV = free choice of diverse diet components: chicory, ryegrass, plantain, and alfalfa; VAR = free choice of plantain and ryegrass from 0700 – 1600 h and chicory and alfalfa between 1600 – 0700 h.; Mean ± Standard error of the mean.

<sup>3</sup> t-test P-value.

Note: Hexanoic and lactic acid were not included as the amounts present were below the detection limit gas chromatogram.



**Table 6.6** Observed behavioral differences within daylight hours of ram lambs fed a SFA, DIV, or VAR diet on day 9 and 20. The values reported in this table are least-squares means  $\pm$  the standard error of the mean for the proportion of time spent doing a specific behavior.

Behavior, % of time <sup>3</sup>	Treatments (TRT) <sup>1</sup>			SEM <sup>4</sup>	P-value <sup>2</sup>		
	SFA	DIV	VAR		TRT	Time	TRTxTime
<b>Eating</b>							
Morning	45.05 <sup>a</sup>	38.27 <sup>b</sup>	31.94 <sup>c</sup>	2.03	<0.01	<0.01	0.29
Afternoon	54.53 <sup>a</sup>	47.75 <sup>b</sup>	41.42 <sup>c</sup>				
Total	50.49 <sup>a</sup>	44.39 <sup>b</sup>	38.20 <sup>c</sup>				
<b>Ruminating</b>							
Morning	28.96 <sup>b</sup>	28.71 <sup>b</sup>	33.53 <sup>a</sup>	1.74	0.03	0.01	0.53
Afternoon	24.50 <sup>b</sup>	24.50 <sup>b</sup>	29.07 <sup>a</sup>				
Total	26.72 <sup>b</sup>	26.91 <sup>b</sup>	31.24 <sup>a</sup>				
<b>Idle</b>							
Morning	23.86 <sup>b</sup>	31.37 <sup>a</sup>	32.48 <sup>a</sup>	2.40	<0.01	0.42	0.20
Afternoon	21.73 <sup>b</sup>	29.24 <sup>a</sup>	30.35 <sup>a</sup>				
Total	22.75 <sup>b</sup>	28.67 <sup>a</sup>	30.36 <sup>a</sup>				
<b>Position, % of time</b>							
<b>Lying</b>							
Morning	44.78 <sup>b</sup>	52.62 <sup>a</sup>	57.88 <sup>a</sup>	2.97	<0.01	<0.01	0.72
Afternoon	35.26 <sup>b</sup>	42.80 <sup>a</sup>	44.95 <sup>a</sup>				
Total	38.77 <sup>b</sup>	46.56 <sup>a</sup>	49.75 <sup>a</sup>				
<b>Standing</b>							
Morning	53.11 <sup>a</sup>	45.37 <sup>b</sup>	41.33 <sup>b</sup>	3.62	<0.01	<0.01	0.38
Afternoon	65.05 <sup>a</sup>	57.30 <sup>b</sup>	53.27 <sup>b</sup>				
Total	61.54 <sup>a</sup>	53.52 <sup>b</sup>	50.13 <sup>b</sup>				

<sup>1</sup> SFA = single forage diet of alfalfa; DIV = free choice of diverse diet components: chicory, ryegrass, plantain, and alfalfa; VAR = free choice of plantain and ryegrass from 0700 – 1600 h and chicory and alfalfa between 1600 – 0700 h.

<sup>2</sup> t-test P-value.

<sup>3</sup> Morning (0700 to 1200 h on day 9 and 0734 to 1200 h on day 20); Afternoon (1200 to 2010 h on day 9 and 1200 to 1942 h on day 20).

<sup>4</sup> SEM = standard error of the mean

<sup>a-c</sup> Means in a row without similar superscripts differ between treatments at each time ( $P \leq 0.05$ ).

**Table 6.7** Observed behavioral differences within daylight hours of ram lambs fed a SFA, DIV, or VAR diet on day 9 and 20. The values reported in this table are least-squares means  $\pm$  the standard error of the mean for the proportion of time spent doing a specific behavior.

Behavior, count <sup>3</sup>	Treatments (TRT) <sup>1</sup>			P-value		
	SFA	DIV	VAR	TRT	Time	TRT×Time
<b>Stereotypic</b>						
Morning	6.72 <sup>a</sup> $\pm$ 1.20	1.55 <sup>b</sup> $\pm$ 0.42	0.35 <sup>c</sup> $\pm$ 0.16	<0.01	<0.01	<0.01
Afternoon	6.50 <sup>a</sup> $\pm$ 1.17	3.59 <sup>b</sup> $\pm$ 0.76	5.10 <sup>ab</sup> $\pm$ 0.96			
Total	13.22 <sup>a</sup> $\pm$ 2.17	5.14 <sup>b</sup> $\pm$ 0.99	5.45 <sup>b</sup> $\pm$ 1.01	<0.01	—	—
<b>Grooming</b>						
Morning	3.79 <sup>b</sup> $\pm$ 0.38	4.37 <sup>b</sup> $\pm$ 0.43	5.32 <sup>a</sup> $\pm$ 0.48	<0.01	<0.01	0.70
Afternoon	8.14 <sup>b</sup> $\pm$ 0.67	9.38 <sup>b</sup> $\pm$ 0.78	11.40 <sup>a</sup> $\pm$ 0.81			
Total	11.93 <sup>b</sup> $\pm$ 0.92	13.75 <sup>b</sup> $\pm$ 1.07	16.72 <sup>a</sup> $\pm$ 1.09	<0.01	—	—

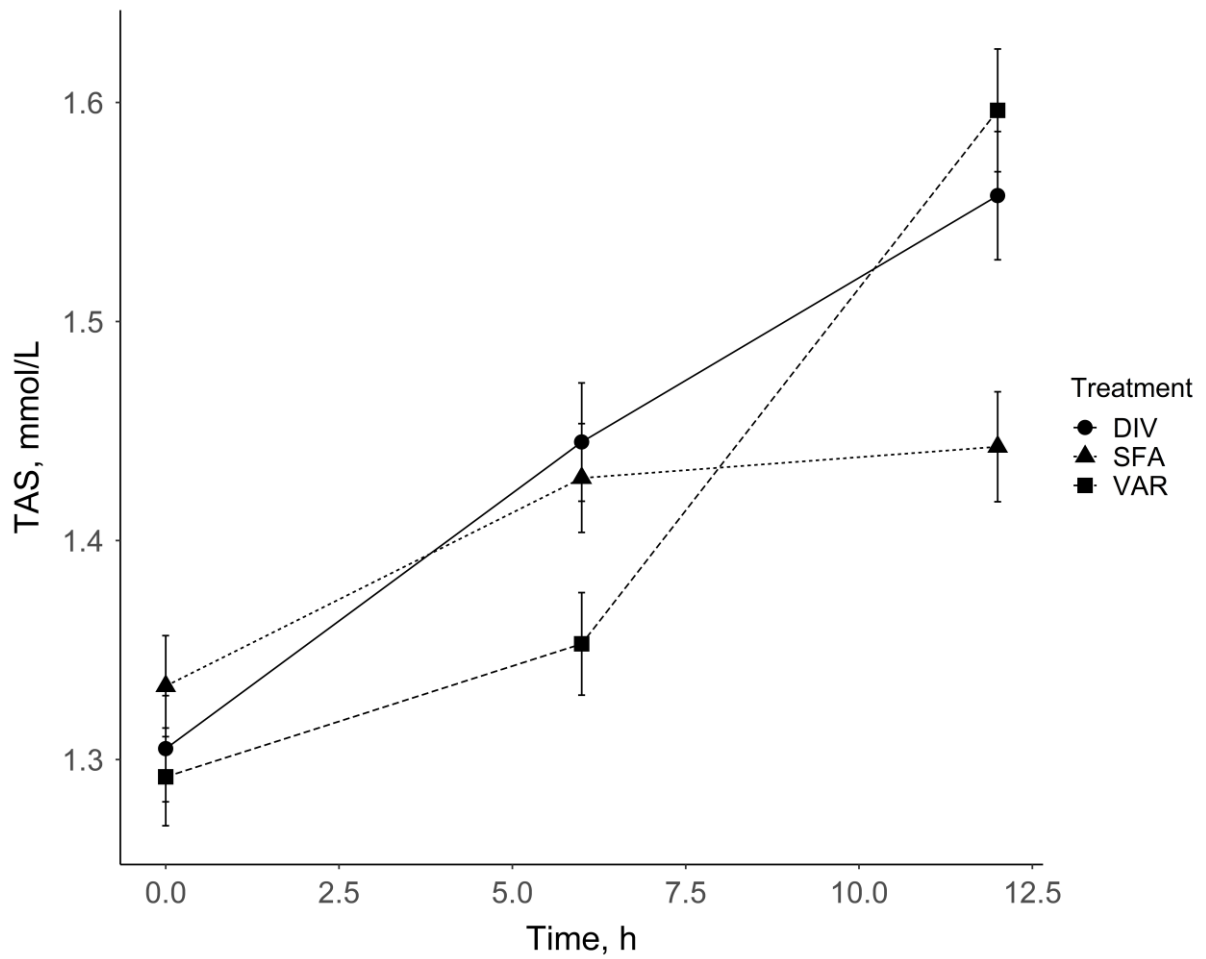
<sup>1</sup> SFA = single forage diet of alfalfa; DIV = free choice of diverse diet components: chicory, ryegrass, plantain, and alfalfa; VAR = free choice of plantain and ryegrass from 0700 – 1600 h and chicory and alfalfa between 1600 – 0700 h.

<sup>2</sup> t-test P-value.

<sup>3</sup> Morning (0700 to 1200 h on day 9 and 0734 to 1200 h on day 20); Afternoon (1200 to 2010 h on day 9 and 1200 to 1942 h on day 20).

<sup>4</sup> SEM = standard error of the mean

<sup>a-c</sup> Means in a row without similar superscripts differ between treatments at each time ( $P \leq 0.05$ ).



**Figure 6.1** Total anti-oxidant status (TAS) of ram lambs fed a DIV (free choice of diverse diet components: chicory, ryegrass, plantain, and alfalfa), SFA (single forage diet of alfalfa), or VAR (free choice of plantain and ryegrass from 0700 – 1600 h and chicory and alfalfa between 1600 – 0700 h) diets at three time points over the day [09:30 (0 h), 15:30 (6 h), and 21:30 (12 h)].

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## Chapter 7

# Don't be a sheep - dietary diversity as opposed to ryegrass can reduce oxidative stress experienced by dams at lambing

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### 7.1 Abstract

I determined if providing ewes in the final trimester of pregnancy with dietary diversity (choice from different plants) reduced the oxidative and metabolic stress they experienced and improved lamb birth weights relative to ewes offered only ryegrass. Fifty-four, twin bearing Coopworth ewes [initial live weight (LW) =  $69.82 \pm 1.16$  kg] were provided with a diverse diet [DIV; spatially separated strips of ryegrass (*Lolium perenne* L.), chicory (*Cichorium intybus* L.), plantain (*Plantago lanceolata* L.), red clover (*Trifolium pratense* L.), and alfalfa (*Medicago sativa* L.)] or a ryegrass diet (RYE) for the final third of gestation. The ewes offered DIV birthed heavier lambs ( $5.64 \pm 0.20$  kg) than the RYE-fed ewes ( $5.18 \pm 0.20$  kg;  $P = 0.03$ ). In addition, the DIV ewes had greater ( $P < 0.01$ ) glutathione peroxidase and total antioxidant status, which suggests lower oxidative stress. They also had lower ( $P = 0.01$ ) plasma non-esterified fatty acid concentrations than the RYE fed ewes 24 hr after lambing, which is indicative of reduced metabolic stress. Greater oxidative and metabolic stress during the periparturient period may increase RYE fed ewes risk of disease susceptibility and lead to reduced maternal transmission of antioxidant protection to lambs. Greater oxidative and metabolic stress may jeopardize animal health and wellbeing, thus highlighting the benefit of a diverse diet compared with a ryegrass only diet.

**Key words:** ryegrass, monotony, diversity, stress, lambing

## 7.2 Introduction

Pregnant ewes and their fetuses, experience oxidative stress caused by the increased production of reactive oxygen species (ROS) (Myatt and Cui, 2004; Garrel et al., 2010; Caroprese et al., 2019; Bouroutzika et al., 2020). These ROS are involved with normal pregnancy and developmental processes, such as implantation and embryo development, fetal defense against uterine infections, pregnancy maintenance, and lambing (Caroprese et al., 2019). Excessive production of ROS can have negative developmental effects or result in abortion (Bouroutzika et al., 2020), and several metabolic disorders experienced in the periparturient period have been linked to oxidative stress (e.g. immune dysregulation, mastitis, and metritis) (Lykkesfeldt and Svendsen, 2007; Sordillo and Aitken, 2009). Animals undergo a number of hormonal and metabolic changes during the periparturient period which increase nutritional demands and stress (Goff and Horst, 1997; Sordillo and Mavangira, 2014). Further, nutritional stress (e.g. excessive fermentable carbohydrates) also may induce oxidative stress (Seyidoglu and Aydin, 2020; Beck et al., 2021). Metabolic, oxidative, and physiological stress are closely related and are thought to act in a mutual reinforcement cycle (Ando and Fujita, 2009; Beck and Gregorini, 2020). Diets that exacerbate nutritional imbalances could be elevating stress and causing greater metabolic and physiological issues as animal's transition from non-lactating to lactation.

Repeated allocation of a single dietary material may induce such nutritional imbalances, as some nutrients are deficient and others are in excess relative to their individual requirements, and thereby compromise animal production, health, and welfare (Ralphs et al., 1995; Provenza et al., 2007; Hogan and Phillips, 2008; Gregorini et al., 2017; Beck and Gregorini, 2020; Beck and Gregorini, 2021). Such repeated diet allocations are frequently implemented in temperate pastoral systems to fulfill basic nutritional requirements and to ease pastoral management. Offering animal's choice from taxonomically diverse diets affords animals the opportunity to select plant combinations that meet their nutrition and therapeutic needs, while negating nutrients that are in excess or that are causing malaise (Villalba et al., 2010), thereby reducing the stress experienced by animals. The objective of this experiment was to determine if providing dietary diversity in the final trimester of pregnancy would reduce the oxidative and metabolic stress experienced by ewes and improve lamb birth weights relative to ewes offered a monotony of ryegrass. We hypothesized that providing animals with dietary diversity would reduce metabolic and oxidative stress experienced by ewes during lambing and improve lamb birth weights relative to ewes offered a monotony of ryegrass.

## 7.3 Materials and Methods

The study was conducted at the Johnstone Memorial Laboratory at Lincoln University (43°38'57"S, 172°27'01"E), according to the methods approved by the Lincoln University Animal Ethics Committee (AEC 2019-34A) prior to experiment initiation.

### 7.3.1 Animal Management and Dietary Treatments

This experiment was conducted between 16 July 2020 and 23 September 2020, totaling 69 days. Twin bearing, Coopworth Ewes (n= 54) in the last third of gestation were stratified by initial live weight (LW) ( $69.8 \pm 1.16$  kg; Mean  $\pm$  SEM). Within stratification, ewes were randomly assigned to treatments: a diverse diet (DIV) or a ryegrass only diet (RYE). The last third of gestation was targeted as approximately 90% of fetal growth occurs during this time (Redmer et al., 2004; Pillai et al., 2017). The animals offered the DIV diet had free access to spatially separated strips of ryegrass (*Lolium perenne* L.), chicory (*Cichorium intybus* L.), plantain (*Plantago lanceolata* L.), red clover (*Trifolium pretense* L.), and alfalfa (*Medicago sativa* L.). Animals had free access to water from a trough at all times. Animals strip grazed and were allocated fresh herbage every 7 days. Weekly pre-grazing quadrat measurements were collected by hand-clipping forage within a 0.25-m<sup>2</sup> quadrat within 3 locations per forage species. Pre-grazing forage mass was used to determine DM availability, so that the quantity of DM allocated could be altered weekly to match the changing metabolizable energy (ME) requirements of the pregnant ewes, following the equations of Rattray et al. (2007). Pre-grazing herbage snip cuts were taken for chemical composition and nutritive value analysis every two weeks. Between 16 July and 10 August all ewes were supplemented with 300 g/head of crushed barley grain to supplement herbage and meet animal energy demands. On average, ewes were provided with  $1.80 \pm 0.12$  kg herbage DM/head per d throughout the study, with no differences between treatments for average DM (P = 0.93) or ME (P = 0.86) allocated.

### 7.3.2 Herbage Establishment

The experimental area was comprised of three large paddocks, each of which was split in half creating six paddocks in total, with three planted in ryegrass and three planted as separated strips of equal area of chicory, plantain, alfalfa, red clover, or ryegrass; the arrangement within paddock was randomized (Figure 7.1). Before planting in October 2019, paddocks were grazed and then prepared for planting by applying glyphosphate (Weedmaster Ts540; 4 L/ha), fluroxypyr (Starane Xtra Herbicide; 1 L/ha), Carfentrazone-E (Hammer Force; 0.1 L/ha), and Polyalkyleneoxide (Slikka; 0.15 L/ha). The area was ploughed and power harrowed 7 d after spraying. The areas to be planted to red clover, alfalfa, and chicory had Trifluralin (2 L/ha) sprayed and incorporated 2 days prior to planting. Planting occurred on the 26 October 2019, using a direct drill with 7.6 cm row spacing, over 14 days

after paddocks were sprayed out. The drill was calibrated for each forage species to provide a seeding rate of 25, 12, 14, 16, and 14 kg/ha for ryegrass (cv. Legion), chicory (cv. Choice), red clover (cv. Relish), alfalfa (cv. Titan), and plantain (cv. Agritonic), respectively. Once pastures were established and weeds were at the three leaf stage, Dicamba (Kamba 500; 0.4 L/ha) was applied onto ryegrass and plantain pastures and Flumetsulum (Preside; 60g/ha) and mineral oil (Uptake; 1 L/ha) were applied to the chicory, clover, and alfalfa. The area was fertilized with 250 kg di ammonium phosphate approximately one and a half months after planting.

### 7.3.3 Herbage Sampling and Analysis

Snip cut samples were obtained by clipping hand grab samples to ground level at 10 random locations within each forage species in the next area to be grazed. The snip cuts were mixed and sub-sampled into 3 approximately equal parts and were randomly allocated to determine the botanical and morphological composition, DM content, and herbage chemical composition. The botanical sample was sorted according to sown species into stem, leaf, reproductive, weeds, and dead material. The sample used to determine chemical composition (Table 7.1) of herbage was stored at -20°C and then freeze dried and ground to pass through a 1-mm screen by a centrifugal mill (ZM200; Retsch, Haan, Germany). Quadrat samples were collected by hand-clipping a 0.25-m<sup>2</sup> area in 3 locations per forage species to ground level using electric clippers. Dry matter percentage was calculated for both snip-cut and quadrat samples by weighing the sample fresh, followed by oven-drying at 60 °C for 7 d before measuring the sample dry weight.

The nutritive value of herbage samples was determined using near infrared spectrophotometry (NIRS; Model: FOSS NIRS Systems 5000, Maryland, USA). Nutritive values used for NIRS calibration were derived prior to sample analysis for DM (AOAC, 1990; method 930.15), organic matter (OM; 100%-ash%; AOAC, 1990; method 942.05), neutral detergent fiber (NDF; Van Soest et al., 1991), acid detergent fiber (ADF; AOAC, 1990; method 973.18), water soluble carbohydrates (WSC; MAFF, 1986), digestible OM in DM (DOMD), DM digestibility (DMD), OM digestibility (OMD; Lowerth et al., 1975), and crude protein (CP) by combustion (Variomax CN Analyser; Elementar Analysensysteme, Hanau, Germany). The NIRS calibration equations all had R<sup>2</sup> values greater than 0.90 and were within the calibration range. Herbage metabolizable energy (ME) was estimated using the Primary Industries Standing Committee, (2007) equation:

$$[\text{ME (MJ)/kg DM}] = \text{digestible OM in DM, \% (DOMD)} \times 0.16 \quad [1].$$

### 7.3.4 Animal Sampling and Measurements

Ewes were sampled for blood prior to treatment allocation and 24 hr after lambing (average lambing date was 10 September 2020 ± 6 d). Blood samples were obtained by jugular venipuncture.

The ewe's blood samples (~10 ml) were collected directly into a heparinized blood tube (Greiner Bio-One International GmbH, Kremsmünster, Austria). From each blood tube a subsample of whole blood and plasma was taken and stored at -20°C until analysis. Plasma was obtained by centrifuging (Megafuge 1.0R, Heraeus Holding GmbH, Hanau, Germany) the remaining whole blood at 2,300 × g at 4 °C for 15 min. Lambs were weighed 24 h after birth using a bucket and a handheld scale (Rapala RDS50, Helsinki, Finland) at the same time the ewes were sampled.

Glutathione peroxidase (GPx) content of the whole blood samples was determined using an enzymatic based protocol (RANSEL; Cat. No. RS504) and a clinical analyzer (Randox Rx Daytona, Crumlin, Co. Antrim, UK). Plasma total antioxidant status (TAS; Cat. No. NX2332) and non-esterified fatty acid (NEFA; Cat. No. FA115) were analyzed as per the instructions of their respective Randox kit manual using a clinical analyzer (Randox Rx Daytona mlin, Co. Antrim, UK).

### 7.3.5 Statistical Analysis

Statistical analysis was conducted using R (R Core Team, 2018, v.3.6.0). The ewe weights were normally distributed ( $P > 0.10$ ; Shapiro-Wilk test) and had homogenous variance ( $P > 0.10$ ; Bartlett's test), thereby meeting the assumptions of an analysis of variance (ANOVA), and were analyzed using the 'aov' function. Other normally distributed data (e.g. TAS and GPx) were analyzed with the 'lmer' function and non-normally distributed data (i.e. lamb birth weight and NEFA) were analyzed with the 'glmer' function with the distribution used for the model selected based on the qq-plots of the residuals, which was determined to be a Gamma distribution. The 'lmer' and 'glmer' models included day lambed as a random factor and dietary treatment as fixed effects (R Core Team, 2018). Pearson's correlation co-efficient between ewe weight and lamb birth weight was determined using the cor.test function of R using the ewe weight 26 d prior to the average lambing date. Least squares means were generated using the 'emmeans' package (Lenth, 2018). Forage cover and nutritive quality were analyzed using the 'glm' function, with repeated measures for fixed-effects.

## 7.4 Results

The herbage had different nutrient (Table 7.1) and botanical composition (Table 7.2). Chicory and plantain had greater ME than alfalfa ( $P < 0.05$ ), none of which were different to either ryegrass or red clover ( $P > 0.05$ ). The DM content of the ryegrass and alfalfa, which were not different ( $P > 0.05$ ), were greater than that of chicory, plantain, and red clover. Plantain had a lower DM content ( $P < 0.05$ ) than ryegrass and alfalfa, which was intermediate and not different ( $P > 0.05$ ) to either chicory or red clover. The WSC content of alfalfa was lower than that of the chicory ( $P < 0.05$ ); however, there were no other differences between the WSC content of herbage. The CP content of the alfalfa and red clover ( $P > 0.05$ ) were greater than that of chicory, plantain, and ryegrass ( $P <$



0.05) and the CP content of chicory was greater than that of plantain and ryegrass ( $P < 0.05$ ), which were not different to one another ( $P > 0.05$ ). The NDF content declined from ryegrass, to alfalfa, to plantain and red clover ( $P > 0.05$ ), to chicory ( $P > 0.05$ ). The barley grain offered for the first few weeks had an as feed DM% of 85%, ME of 15.56 MJ/ kg DM, and a CP (%DM) of 10.41. Ryegrass, chicory, red clover, and alfalfa were all in a vegetative state with leafy herbage comprising 76.1, 92.6, 89.3, and 64.8 % of total DM. Plantain was in early reproductive state over the course of the trial, with just 9.4% of plantain DM being comprised of reproductive stem.

Twenty six  $\pm$  6 d prior to the average lambing date, the DIV ewes ( $78.71 \pm 1.63$  kg) did not differ in weight from the RYE ewes ( $75.76 \pm 1.60$  kg;  $P = 0.20$ ). There was no difference in lambing date between treatments ( $P = 0.95$ ), and as the ewes were all mated at the same time, which suggests there is no difference in gestation length caused by treatments. The birth weight of lambs from the DIV ewes ( $5.64 \pm 0.20$  kg) was 9% greater than lambs born to RYE fed ewes ( $5.18 \pm 0.20$  kg;  $P = 0.03$ ; Table 7.3). Twenty-four hr after lambing, the DIV ewes GPx concentration ( $16.17 \pm 0.50$  U/mL) was 35% greater ( $P < 0.01$ ) than that of the RYE ewes ( $11.98 \pm 0.50$  U/mL). In addition, the TAS concentration of the DIV treatment ( $1.38 \pm 0.02$  mmol/L) was 8% greater than that of the RYE ewes ( $1.28 \pm 0.02$  mmol/L;  $P < 0.01$ ). Further, the NEFA concentrations of the RYE ewes ( $0.68 \pm 0.16$  mmol/L) was 74% greater than DIV ewes ( $0.38 \pm 0.07$  mmol/L;  $P = 0.01$ ).

## 7.5 Discussion

We hypothesized that providing dietary diversity (i.e. DIV) would reduce the oxidative and metabolic stress experienced by ewes at parturition and improve the birth weight of lambs compared with ewes grazing ryegrass (i.e. RYE). The results support this hypothesis. The kg DM /ewe per d and ME MJ/ewe per d of the diets offered were not different, thereby allowed for comparison to test this hypothesis.

Ewes offered DIV had heavier lambs at birth than ewes fed RYE. This differs with the results of Hutton et al. (2011) and Kenyon et al. (2010), who reported no difference in lamb birth weight from ewes fed either a ryegrass diet or offered a herb and legume mixed sward (chicory, plantain, red and white clover). This is despite similarities in pregnant ewe weight between Kenyon et al. (2010), Hutton et al. (2011) and the present study; there no difference in ewe weight by treatment at day 132 of pregnancy by Kenyon et al. (2010), day 140 by Hutton et al. (2011), and day 126 of pregnancy ( $26 \pm 6$  d prior to lambing) of our study. Ewe weight and lamb birth weight were not correlated ( $P = 0.14$ ) and the percentage of lamb birthweight as a percentage of ewe weight was not different ( $P = 0.23$ ), which was also reported by Fogarty et al. (1992), who applied treatments of low and high nutrition to ewes during mid-pregnancy. Greater lamb birth weight is associated with decreased mortality (Morel et al., 2009). Lighter birth weight lambs are more likely to succumb to

mortality caused by starvation and exposure (Fogarty et al., 1992), while overly heavy birth weights are associated with dystoica (Horton et al., 2018).

A potential cause of this difference in lamb weight may be related to RYE-fed ewes having a lower feed conversion efficiency due to lower digestibility. Garrett et al. (2021) fed similar forage diets and found that feed conversion efficiency of ram lambs offered a multi-forage diverse diet was 36% greater than that of lambs grazing ryegrass only. In the current study if there was such a marked difference in feed conversion efficiency we would have expected a difference in ewe body weight. In addition, both under- and over-nutrition can reduce fetal growth rates (Robinson, 1977; Russel et al., 1981), making it difficult to ascertain the cause of the RYE lambs lower birth weight, considering the ewes were allocated enough quality feed to meet their estimated nutritional needs. Under-nutrition through incidental restriction (cessation of eating as a nutrient or plant secondary compound reaches toxic levels, while other nutritional needs remain unfulfilled) or over nutrition through incidental augmentation (consumption continues to meet other nutritional needs, despite one nutrient having been consumed at excessive levels) may have occurred (Raubenheimer, 1992; Bailey and Provenza, 2008). Offering animals a range of taxonomically, and implicitly phytochemically, diverse feeds allows the animal to choose plant combinations that meet their nutritional and medicinal needs, while potentially negating nutrients that are causing malaise or toxicity (Villalba et al., 2010). Perhaps, the ability of ewes to better meet their individual nutritional and nutraceutical needs on the DIV diet contributed to their greater lamb size and improved antioxidant status post lambing.

The DIV ewes had improved antioxidant status and reduced oxidative and metabolic stress as evidenced by the greater GPx, TAS, and lower NEFA. Plasma TAS describes the total balance between oxidizing species and antioxidants and therefore may be more representative of the antioxidant-to-oxidant balance than a single antioxidant (Ghiselli et al., 2000). Elevated TAS is indicative of reduced oxidative stress or increased capacity to cope with oxidative stress. Further, increased GPx and reduced NEFA seen in the DIV ewes further supports the DIV ewes having lower oxidative stress.

The DIV ewes had a much lower NEFA concentration. This is unexpected considering they had a numerically greater LW, as greater live weight has been associated with elevated NEFA concentrations following parturition (Rukkwamsuk et al., 1998; Bernabucci et al., 2005). The mobilization of fat stores indicated by elevated NEFA has been associated with increased oxidative stress (Sordillo and Aitken, 2009; Sordillo and Mavangira, 2014; Li et al., 2016). While plasma GPx can be interpreted as a marker of oxidative stress, it is useful to consider alongside other markers, as elevated GPx levels can also be indicative of the presence of a stressor (Bernabucci et al., 2002; Beck et al., 2021) or improved antioxidant status due to greater dietary supply of precursor materials (e.g. selenium) (Gerloff, 1992). We believe the latter is true when coupled with the greater TAS and lower

NEFA concentration, which also suggested reduced oxidative stress. Grant and Sheppard (1983) reported that deeper rooting plants can have selenium contents more than three times greater than grasses (see paper for breakdown of vast number of grass species offered) and that alfalfa has particularly high levels of selenium. Another experiment by Harrington et al. (2006) showed greater selenium concentration in plantain and chicory, compared with ryegrass. The greater expected selenium content of the diverse diet components (chicory, plantain, and alfalfa), suggests a greater supply of this precursor mineral, allowing greater antioxidant status of the DIV animals resulting in elevated GPx levels. The cumulative results of elevated TAS and GPx, and reduced NEFA concentrations of the DIV ewes provide evidence of reduced oxidative stress 24 h after lambing compared to the RYE treatment.

Increased oxidative stress during the periparturient period contributes to increased disease susceptibility (Mikulková et al., 2020), with links to several metabolic disorders (e.g. immune dysregulation, mastitis, and metritis) (Lykkesfeldt and Svendsen, 2007; Sordillo and Aitken, 2009). Single forage diets may increase animals' susceptibility to disease compared to animals consuming a diverse diet. Increased antioxidant defense in dams may be transmitted *in utero* or in early life to offspring (Beck et al., Unpublished; Nieto et al., 2010a; Nieto et al., 2010b). Such maternal transmission of antioxidant defense is hypothesized to reduce the morbidity and mortality of lambs; however, further investigation is required. Ultimately, the current study indicates that the DIV diet fed to ewes in the final third of gestation may enhance their antioxidant status, providing health benefits after a physiologically stressful event, such as lambing.

## **7.6 Conclusions**

Feeding the DIV diet during the final third of gestation reduced the oxidative stress experienced by the ewes at lambing compared to the feeding the RYE diet. Evidence for this was seen in increased TAS and GPx, and reduced NEFA of ewes fed the DIV diet compared with the RYE. Diverse diets fed in the final third of gestation may improve the antioxidant status of the ewes at lambing, which could provide greater protection against transitional diseases and impart greater antioxidant defense to lambs, relative to a monotonous diet of ryegrass.

**Table 7.1** Chemical composition of the herbage composing the single forage perennial ryegrass diet (RYE) or a taxonomically diverse multi-forage choice (DIV) diet of ryegrass, chicory (*Cichorium intybus* L.), alfalfa (*Medicago sativa* L.), plantain (*Plantago lanceolata* L.), and red clover (*Trifolium pretense* L.).

Item <sup>1</sup>	Herbage					SEM <sup>2</sup>
	Ryegrass	Chicory	Plantain	Alfalfa	Red clover	
Cover, kgDM/ha	4204	1774	2313	2322	2293	384
ME, MJ/kg of DM	11.63 <sup>ab</sup>	12.86 <sup>a</sup>	12.57 <sup>a</sup>	10.68 <sup>b</sup>	11.58 <sup>ab</sup>	0.41
DM, % as-is	21.48 <sup>a</sup>	12.67 <sup>c</sup>	15.04 <sup>bc</sup>	21.97 <sup>a</sup>	16.81 <sup>b</sup>	1.22
OM, % DM	90.17 <sup>a</sup>	86.25 <sup>b</sup>	87.78 <sup>b</sup>	91.71 <sup>a</sup>	90.32 <sup>a</sup>	0.79
OMD, % DM	80.29 <sup>b</sup>	90.97 <sup>a</sup>	86.77 <sup>a</sup>	72.14 <sup>b</sup>	80.92 <sup>ab</sup>	2.73
WSC, % DM	20.54 <sup>ab</sup>	22.67 <sup>a</sup>	20.72 <sup>ab</sup>	14.71 <sup>b</sup>	19.24 <sup>ab</sup>	2.86
NDF, % DM	49.00 <sup>a</sup>	13.61 <sup>d</sup>	20.45 <sup>c</sup>	28.79 <sup>b</sup>	20.14 <sup>c</sup>	2.13
ADF, % DM	26.50 <sup>a</sup>	15.66 <sup>c</sup>	17.37 <sup>bc</sup>	25.32 <sup>a</sup>	18.97 <sup>b</sup>	1.16
CP, % DM	14.03 <sup>c</sup>	17.87 <sup>b</sup>	14.66 <sup>c</sup>	25.09 <sup>a</sup>	24.54 <sup>a</sup>	1.00

<sup>a-d</sup> Means in a row with different superscripts are statistically different ( $P < 0.05$ ).

<sup>1</sup> ME, metabolizable energy; DM, dry matter; OM, organic matter; OMD, OM digestibility; WSC, water-soluble carbohydrates; NDF, neutral detergent fiber; ADF, acid detergent fiber; CP, crude protein.

<sup>2</sup> SEM, standard error of the mean.

**Table 7.2** Botanical composition of the herbage composing the single forage perennial ryegrass diet (RYE) or a taxonomically diverse multi-forage choice (DIV) diet of ryegrass, chicory (*Cichorium intybus* L.), alfalfa (*Medicago sativa* L.), plantain (*Plantago lanceolata* L.), and red clover (*Trifolium pretense* L.).

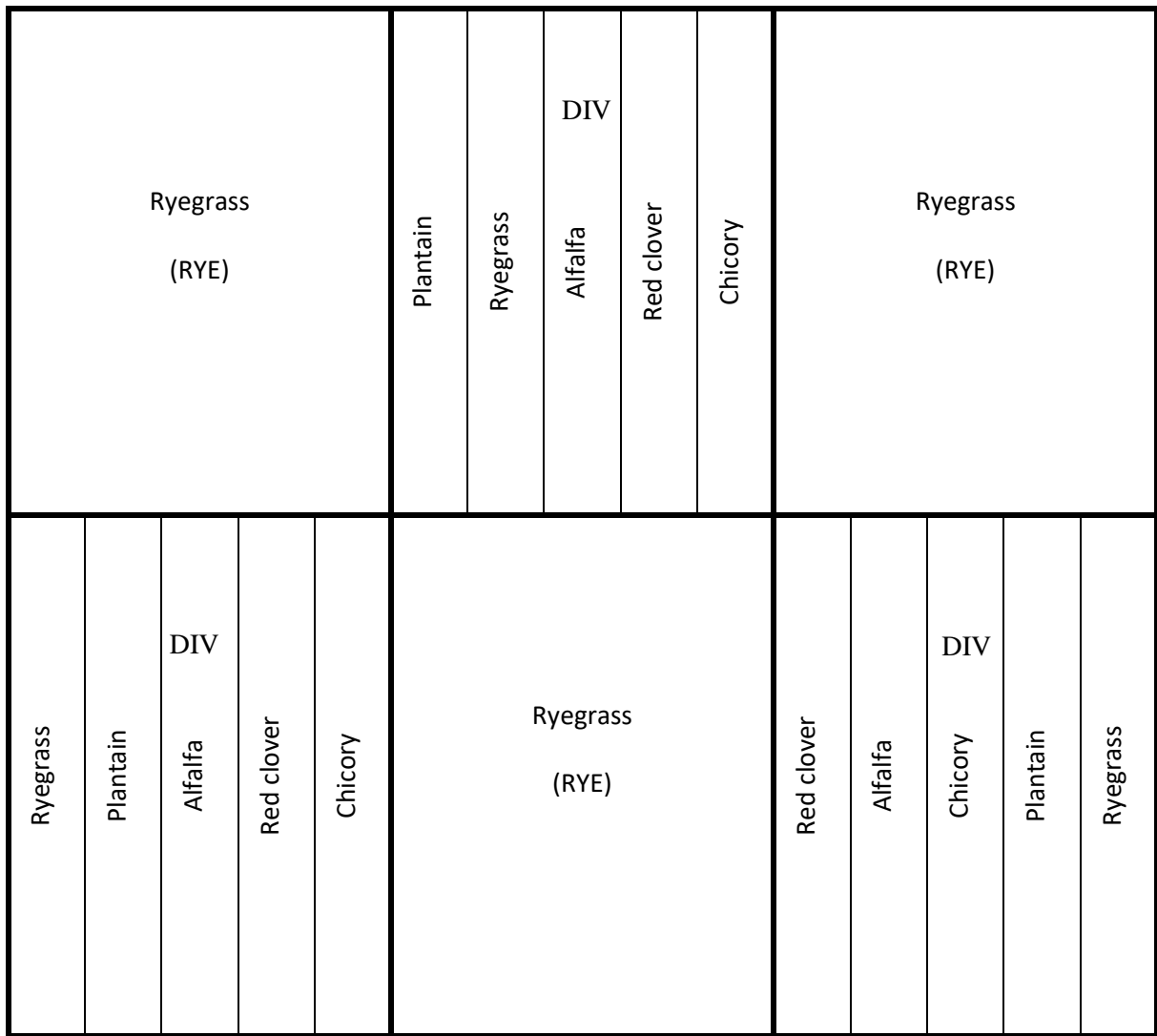
Item <sup>1</sup>	Herbage				
	Ryegrass	Chicory	Plantain	Alfalfa	Red clover
Leaf	76.1 ± 4.0	92.6 ± 6.7	89.3 ± 6.5	64.8 ± 4.6	89.3 ± 6.5
Repro.	8.2 ± 5.8	—	9.4 ± 3.6	—	—
Weed	23.3 ± 5.7	6.4 ± 6.2	1.7 ± 0.9	20.0 ± 5.9	6.8 ± 3.9
Dead	23.3 ± 5.7	5.2 ± 1.4	4.3 ± 1.2	14.9 ± 4.3	3.7 ± 1.0

<sup>1</sup>Repro. = reproductive stem or flower

**Table 7.3** Animal measurements for sheep allocated either a single forage perennial ryegrass diet (RYE) or a taxonomically diverse multi-forage choice (DIV) diet of ryegrass, chicory (*Cichorium intybus* L.), alfalfa (*Medicago sativa* L.), plantain (*Plantago lanceolata* L.), and red clover (*Trifolium pretense* L.) 24 hr after lambing.

Item <sup>1</sup>	Treatment		
	RYE	DIV	P- value
Lamb birth weight, kg	5.18 ± 0.20	5.64 ± 0.20	0.03
GPx, U/mL	11.98 ± 0.50	16.17 ± 0.50	<0.01
TAS, mmol/L	1.28 ± 0.02	1.38 ± 0.02	<0.01
NEFA, mmol/L	0.68 ± 0.16	0.38 ± 0.07	0.01

<sup>1</sup> GPx, glutathione peroxidase activity of the whole blood; TAS = total antioxidant status of plasma; NEFA, Non-esterified fatty acids content of plasma



**Figure 7.1** Experimental area and paddock layout grazed by ewes with access to only Ryegrass (*Lolium perenne* L.; RYE) or ewes allocated a diverse diet (DIV) of spatially separated strips of equal area, of chicory (ryegrass, chicory (*Cichorium intybus* L.), alfalfa (*Medicago sativa* L.), plantain (*Plantago lanceolata* L.), and red clover (*Trifolium pretense* L.).

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## Chapter 8

### From the get-go: dietary exposure in utero and in early life alters dietary preference in later life

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#### 8.1 Abstract

The objectives were to determine the effect of *in utero* and early-life dietary exposure to a diverse diet or a perennial ryegrass (*Lolium perenne* L.) diet on grazing behavior and dietary neophobia of lambs ~3.5 months post-weaning (weaned at 6 weeks of age). Sixty-four Coopworth lambs (152.5 ± 1.4 d of age; mean ± SEM,) were used. Twenty lambs had previous *in utero* exposure to the diverse diet (INDIV) and another 20 had *in utero* and early life (from birth to weaning at 42 ± 2 d of age) exposure to the diverse diet (ELDIV) of ryegrass, red clover (*Trifolium pratense* L.), plantain (*Plantago lanceolata* L.), chicory (*Cichorium intybus* L.), and alfalfa (*Medicago sativa* L.). Twenty lambs had only *in utero* exposure to ryegrass (INRYE) and the other 20 had early life exposure to ryegrass (ELRYE). Lambs were randomly allocated to one of 16 paddocks (n = 4 per treatment) with 5 lambs per paddock. All paddocks contained equal areas of spatially separated strips of red clover, chicory, alfalfa, plantain, and ryegrass, of which arrangement within the paddock was randomized. Every 5 min from 0620 – 2105 h, trained observers recorded the behavior (grazing, idle, and ruminating) and the location (pasture species) of each lamb. The INRYE and ELRYE lambs tended to ( $P \leq 0.10$ ) and spent more time ( $P < 0.05$ ) grazing within ryegrass than their diverse treatment counterparts, respectively. The ELRYE treatment spent longer grazing in ryegrass, a product of increasing bout number within ryegrass ( $P < 0.05$ ) and the INRYE treatment had longer bouts within ryegrass compared with their diverse treatment counterparts. Further, the INRYE and INDIV treatments spent less time grazing ryegrass than the other treatments ( $P < 0.05$ ). The ELDIV and ELRYE treatments in general had a reduced latency to graze pasture species compared to the other treatments. Further, all lambs ate a mix of the forages offered. Exposure *in utero* and early in life affected the preferences

of lambs exposed to diverse species after weaning. Such effects were still seen ~3.5 months after weaning and differences between treatments were more prominent in lambs exposed in the early life treatment group compared with the *in utero* treatments. Exposure to ryegrass *in utero* and early in life exposure pre-disposed animals to prefer ryegrass later in life, while diverse diet exposure appeared to reduce dietary neophobic behavior.

**Key words:** Dietary neophobia; dietary preference; foraging ecology; ruminant livestock.

## 8.2 Introduction

The introduction of novel feeds and the incorporation of unfamiliar feeds into a feeding regime or landscape can have negative effects on animals dry matter intake, production, the environment, and the financial viability of practices, as ruminants display neophobic (fear of new) behaviors (Launchbaugh and Provenza, 1991). Neophobic behaviors are thought to be a mechanism to allow for ingestion of a small sample of feed to determine post-ingestive consequences (e.g., toxicity) and form associations with pre-ingestive (i.e., oro-sensorial) cues for identification during subsequent interactions (Provenza, 1995). This theory is supported by sheep grazing within an unfamiliar rangeland type spending more time grazing and consuming less forage compared with animals familiar with the rangeland (Arnold, 1970).

Neophobic behaviors can result in over-grazing of familiar feeds, resulting in damage (e.g., soil compaction) of areas where these are located. In contrast, unfamiliar feeds remain underutilized despite their strategic incorporation into grazing systems or swards (e.g., anthelmintic properties, feeding value, or environmental protective properties) or inclusion in feed budgets (Launchbaugh and Howery, 2005; Beck and Gregorini, 2020; Beck and Gregorini, 2021). As such, neophobic behaviors displayed as reduced intake and increased sampling represent a potentially significant economic loss, as this would decrease productivity and increase time to finishing and associated operating cost (Launchbaugh and Howery, 2005; Beck and Gregorini, 2021). Further, many animals encounter novel feeds and forages at weaning, when they are placed into systems for finishing, potentially creating an extensive issue for production systems (Hatfield et al., 1992; Ortega-Reyes et al., 1992). Early life experience (*in utero*, through milk ingestion, social interactions [dams and peers], and exposure to feeds) is a major determinant of behavioral patterns and preference later in life (Arnold and Maller, 1977; Catanese et al., 2012; Villalba et al., 2012; Beck and Gregorini, 2021). For example, Hai et al. (2014) reported greater post-weaning intake of *Chromolaena odorata* by goat kids that been exposed to it *in utero* through their dams ingestion, compared with those without any experience. Therefore, providing a diverse diet during this period could pre-program animals with a base knowledge of plants, reducing the incidence of the negative consequences associated with neophobic behaviors.

Despite the knowledge of early life experience impacting intake and performance in later life, diets allocated during this period remain relatively homogenous and repetitive (e.g., pastoral ryegrass-based diets). Recent studies have shown that providing diverse diets to sheep at finishing can increase dry matter intake (DMI) and productivity, while reducing environmental impact compared with monotonous single forage diet (Garrett et al., 2021). Therefore, we were interested in exploring and isolating *in utero* and early-life effects of exposure to a diverse diet compared with a ryegrass diet and how previous exposure influences preference for diverse pastures if encountered again later in life. Currently, there is a lack of knowledge on how *in utero* and early life exposure to repetitive single forage diets and diverse diets might influence dietary preferences and grazing behaviors later in life. Further, we were interested in determining if animals prefer to comprise a diverse diet or if given the opportunity will consume only the forage they prefer. The first objective was to determine if *in utero* experience to a diverse multispecies or single species ryegrass diet altered forage preference and neophobic behavior of lambs after weaning. The second objective was to determine if early life experience to a diverse or a ryegrass diet altered preference and reduced neophobic behavior after weaning. A third objective was to determine if *in utero* and early-life dietary exposure to these diets caused animals to comprise a diet of mixed species. We hypothesized that *in utero* and early-life exposure to either diverse and ryegrass only diets would alter animal's preference towards other species when first encountering them after weaning. We further hypothesized that differences in preference between diverse and ryegrass diets would be greater for animals with the whole early life (*in utero* and alongside dam) exposure compared with just *in utero* exposure. Finally, we hypothesized that all treatments would eat a mixed diet including all of the species available to graze.

### **8.3 Materials and Methods**

The study was conducted at the Lincoln University Johnstone Memorial Laboratory (43°38'57"S, 172°27'01"E), as per methods approved by the Lincoln University Animal Ethics Committee (AEC 2019-34A).

In brief, lambs were exposed to a diverse diet (DIV) comprised of 5 forage species or a ryegrass diet (RYE) *in utero* (IN) or both *in utero* and early life (EL). Three and a half months after weaning, lambs were introduced to spatially separated strips of diverse pasture species and their behavior, latency to graze (time spent taken to first graze each species), and time spent grazing in each species were recorded.

### 8.3.1 Herbage Establishment, Sampling, and Analysis

The preference paddocks were strips of areas within larger blocks, separated into five strips of equal area of red clover, chicory, alfalfa, plantain, or ryegrass arranged randomly within blocks. Blocks were prepared for planting by biological defoliation followed by application of glyphosphate (Weedmaster Ts540, Nufarm, Auckland, NZ; 4 L/ha), fluroxypyr (Starane Xtra Herbicide, Dow AgroSciences; New Plymouth, NZ; 1 L/ha), Carfentrazone-E (Hammer Force, FMC, Auckland, NZ; 0.1 L/ha), and Polyalkyleneoxide (Slikka, Etec Crop Solutions, Auckland, NZ; 0.15 L/ha). The area was ploughed and power harrowed 7 days after spraying. The areas selected to be planted with clover, alfalfa, and chicory had Trifluralin (Genfarm Trifluralin 480 Selective Herbicide, Nutrien Ag Solutions, New South Wales, AUS; 2 L/ha) applied and incorporated appropriately. On 26 October 2019, 14 days after the paddocks were originally sprayed, planting occurred using a direct drill with 7.6 cm row spacing. The drill was calibrated to each forage species to provide a seeding rate of 25, 12, 14, 16, and 14 kg/ha for ryegrass (cv. Legion), chicory (cv. Choice), red clover (cv. Relish), alfalfa (cv. Titan), and plantain (cv. Agritonic), respectively. Once established Dicamba (Kamba 500, Nufarm, Auckland, NZ; 0.4 L/ha) was applied onto the ryegrass and plantain containing areas and Flumetsulum (Preside, Dow AgroSciences, New Plymouth, NZ; 60g/ha) and mineral oil (Uptake, Dow AgroSciences, New Plymouth, NZ; 1 L/ha) were applied to the chicory, clover, and alfalfa areas. All forages were fertilized with 250 kg di ammonium phosphate.

On the 9th February 2021 quadrats and snip cuts were collected in each paddock to determine the quantity (DM/ha), DM percentage, chemical composition, and botanical composition of the herbage. These samples were repeated after grazing on 12th February 2021, to determine the quantity of residual herbage and to obtain representative samples. The snip cut samples were taken from 10 random locations within each species strip. These samples were mixed and sub-sampled in three equal parts to determine the botanical composition, DM, and herbage chemical composition. The botanical composition was determined by sorting the representative sub-samples into sown species stem, leaf, and reproductive and also into weeds and dead material, before drying at 60°C for 7 days. Herbage chemical composition (Table 8.1) was determined after storing samples at -20°C, freeze-drying, and grinding (ZM200; Retsch, Haan, Germany; 1mm screen). The sample used to determine DM percentage was weighed, dried for 7 days at 60°C, and re-weighed dry. This drying method was also used on quadrat samples, which consisted of an area of 0.25 m<sup>2</sup> of herbage cut to ground level with electric clippers to determine the DM per hectare.

Herbage chemical composition was determined using near infrared spectrophotometry (NIRS; Model: FOSS NIRS Systems 5000, Maryland, USA). The NIRS calibration used chemical composition values derived prior to sample analysis for DM (AOAC, 1990; method 930.15), organic

matter (OM; 100%-ash%; AOAC, 1990; method 942.05), neutral detergent fiber (NDF; Van Soest et al., 1991), acid detergent fiber (ADF; AOAC, 1990; method 973.18), water-soluble carbohydrates (WSC; MAFF, 1986), digestible OM in DM (DOMD), DM digestibility (DMD), and OM digestibility (OMD; lowerth et al., 1975), and crude protein (CP) by combustion (Variomax CN Analyser; Elementar Analysensysteme, Hanau, Germany). The R<sup>2</sup> values for the NIRS calibration equations were all greater than 0.90 and were within the calibration range. The herbage ME was estimated using the Primary Industries Standing Committee (2007) equation as follows:

$$\text{ME (MJ/kg DM)} = \text{DOMD} \times 0.16 \quad [1].$$

### 8.3.2 Animal Management and Treatments

The treatment was designed as a 2 × 2 factorial of diet and duration of exposure to the diet. Sixty-four lambs were born to ewes (selected as all twin bearing) provided either the DIV or RYE diet. The DIV ewes grazed spatially separated strips of perennial ryegrass, red clover (*Trifolium pratense* L.), plantain (*Plantago lanceolata* L.), chicory (*Cichorium intybus* L.), and alfalfa (*Medicago sativa* L.) in the final third of gestation, and the RYE sheep grazed a monoculture of perennial ryegrass in the final third of gestation. Twenty-four hours after lambing, the twin bearing dams were left with one lamb, which remained with them on their respective diet until weaning 6 weeks later, thereby providing early life experience to their respective diets. Hereon these lambs will be referred to as ELDIV (early life experience to DIV) and ELRYE (early life experience to RYE). Lambs removed from dams were bottle raised and had access to ryegrass until weaning, from hereon they will be referred to as INDIV (*in utero* exposure to DIV) and INRYE (*in utero* exposure to RYE). From weaning until the start of the preference testing all lambs were kept on a ryegrass diet. The IN and EL treatments were balanced for lamb sex. Half of the lambs were rams and the other half were ewes. At the start of the preference treatment there was no difference in live weight between the two *in utero* treatments (27.64 ± 0.98 kg; P = 0.93) or the two early life treatments (31.90 ± 0.95 kg; P = 0.41). However, IN lambs (27.64 ± 0.98 kg) were lighter than EL lambs (31.90 ± 0.95 kg, P < 0.01). Examination of twins allowed us to determine if there was an *in utero* effect or if any effect occurred later through early life (*in utero*, transmission through milk, social learning, and personal) experience. Further, as preference testing was conducted in a manner that allowed animals to comprise their diet of a single feed if desired, it allowed us to determine if lambs with a range of diet histories preferred to mix their diets.

The ewes had access to their respective diets from 16th July 2020 until weaning (~20 October 2020), 6 weeks ± 2 days after lambing. All sheep had free access to water throughout the experiment. Treatment swards were rotationally grazed with a residence time of 7 days per paddock. From the 16th July to 10th August, herbage from the two treatments was supplemented with



crushed barley grain (300 g/ head). The area allocated and thereby the dry matter (DM) allocated was set to match the metabolizable energy (ME) requirements of the ewes throughout pregnancy, as per the equations of Rattray et al. (2007) and the DM/ha information obtained from quadrat cuts of herbage. The average herbage allocation was  $1.80 \pm 0.12$  kg DM/hd per d, and no differences between treatments were detected for the average DM ( $P = 0.93$ ) or ME allocated ( $P = 0.86$ ).

Lambs were grouped by treatment and sex and were randomly allocated to one of 16 paddocks ( $n = 4$ ), with five lambs per paddock. Herds of animals require a minimum of three animals per paddock to display normal grazing behaviors (Penning et al., 1993). The stocking density for the preference testing was set at 130 m<sup>2</sup> /sheep per d to ensure that lambs had adequate forage to compose their diet of a single forage if they so choose. The evening prior (9 February 2021) to the first day of observations lambs (age =  $152.5 \pm 1.4$  d) were penned within a ryegrass paddock near their respective experimental paddock. Although basal diet, in this case ryegrass, can affect preference, feed selection for subsequent meals, and neophobia (Wang and Provenza, 1996; Early and Provenza, 1998; Scott and Provenza, 1999) all treatments had exposure to the same basal diet prior to the preference testing, allowing for differences in preference and neophobia to be concluded from their dietary treatment months prior. On the morning of observation day one (10 February 2021) at 0530 h lambs were moved into their experimental paddock for that day. Similarly, on day two of observations (11 February 2021) the animals were given access to a fresh break of herbage at 0530 h.

### **8.3.3 Behavioral Observations**

Grazing behavior and dietary preference of the lambs were determined by scan sampling on the 10th and 11th of February 2021 about 3.5 months after weaning. (Altmann, 1974; Villalba et al., 2015; Beck et al., 2021). Scan sampling was done every 5 min between 0620 – 2105 h, with location (i.e., the strip of forage species located within) and behavior (grazing, ruminating, or idle) recorded for each animal. Trained observers (1 person per 16 lambs) with access to binoculars, recorded the behavior and location of animals, identifiable at a distance by a unique marking given to each sheep with an aerosol marker.

The data were examined as a whole day (Day; 0620 – 2100 h, 880 min). Sheep behavior (grazing, ruminating, and idling) was expressed as the proportion of total observation time spent on that behavior. Additionally, the percent of time spent grazing each species was calculated as a percentage of the total grazing time. Grazing bouts were considered as the time grazing within a specific patch (forage species), while a grazing meal was a cluster of grazing bouts (Gregorini et al., 2006; Beck et al., 2021). The number and duration of grazing bouts were determined by considering

one event and its duration, as the amount of time until the activity was broken by altering behavior or location.

### **8.3.4 Statistical Analysis**

The software R was used for all statistical analyses (R Core Team, 2018, v.3.6.0), significance was considered as  $P \leq 0.05$ , and tendencies were declared at  $P \leq 0.10$ . The treatment design was a  $2 \times 2$  factorial arrangement of diet and duration of exposure, and the preference testing treatment to paddock allocation was completely randomized design. The 'glmer' function of the 'lme4' package was used with a Gamma distribution for all behavior data, except for the distribution of grazing bouts, which used a Poisson distribution (Bates et al., 2015). The animal was considered the observational unit and the experimental unit was considered as the paddock replicate, thereby the animal was used as a random effect. Day and sex were also included as a random effects. Behavioral analysis of the proportion of time spent grazing, ruminating, and idling and the grazing activity data (i.e., grazing bout count and duration) used treatment, day, time, and their interaction as fixed effects. While the proportion of time spent grazing each forage species included treatment, day, time, and their interactions as fixed effects. The forage data were explored with block and paddock as a random effect. Once the model was fit, an analysis of deviance table was composed with the 'Anova' function of the 'car' package to generate a type II Wald Chi-squared test (Fox and Weisberg, 2011). Treatment effects were compared using orthogonal contrasts designed to test differences between 1) the *in utero* treatments (INDIV and INRYE), 2) the early-life treatments (ELDIV and ELRYE), and 3) the *in utero* and early-life treatments. The contrasts were generated using the 'emmeans' package (Lenth, 2018).

## **8.4 Results**

### **8.4.1 Herbage Characteristics**

There was no difference in botanical composition of herbage between treatments, therefore the average botanical composition and sward height data are reported in Table 8.1 as an average for each species. Herbage chemical composition did not differ between treatments ( $P > 0.10$ ; Table 8.2).

### **8.4.2 Grazing Behaviour and Forage Preference**

There were no differences between the *in utero*, early life, or *in utero* vs early life treatments on the percentage of time spent grazing, idle, or ruminating ( $P > 0.10$ ; Table 8.3). The INRYE treatment had approximately four less bouts per day ( $P = 0.04$ ) than the INDIV treatment. Duration

of grazing bouts differed between INDIV and INRYE, with INDIV bouts 9% shorter than INRYE bouts ( $P = 0.02$ ).

The ELDIV treatment spent 10% less of their grazing time in ryegrass than the ELRYE treatment ( $P = 0.05$ ; Table 8.4) and the INDIV tended to spend a lower proportion of time grazing in ryegrass compared with INRYE ( $P = 0.10$ ). Further, the *in utero* treatments spent 7% less time grazing ryegrass than the early life treatments ( $P = 0.02$ ). The INRYE treatment tended to spend 4% more of their time grazing red clover compared with the INDIV ( $P = 0.06$ ). The *in utero* treatments spent a greater percentage of time grazing red clover and chicory compared with the early life treatments ( $P < 0.05$ ). The ELDIV treatment spent 10% more time grazing plantain compared with the ELRYE ( $P = 0.02$ ). The INDIV treatment spent 7% more time grazing alfalfa compared with the INRYE ( $P = 0.03$ ).

In ryegrass, the INRYE treatment tended to have grazing bouts that were 21% longer than the bouts of the INDIV ( $P = 0.06$ ; Table 8.5). The ELRYE treatment had 2 more grazing bouts ( $P = 0.02$ ) within ryegrass than the ELDIV, and the average duration of the ELRYE lambs grazing bouts within ryegrass were 47% longer in duration ( $P = 0.05$ ) than the bouts of the ELDIV. In the ryegrass strip, the early life treatments had 2.6 more grazing bouts and on average all grazing bouts that were 17% longer in duration compared with the *in utero* treatments ( $P < 0.05$ ). *In utero* treatments tended to have one additional grazing bout a day in chicory ( $P = 0.07$ ), and each of the bouts within chicory were on average 12% longer in duration than the bouts of the early life treatments ( $P = 0.05$ ). The diverse treatments had 17% more grazing bouts in alfalfa than the ryegrass treatments. The ELDIV treatment had 94% more grazing bouts within plantain than the ELRYE ( $P = 0.02$ ). The grazing bouts of INRYE in red clover were 39% longer compared with the INDIV ( $P < 0.01$ ).

No treatment effects on the latency to graze in plantain and ryegrass were detected ( $P > 0.10$ ; Table 8.6). The INDIV began grazing chicory 43% sooner ( $P = 0.02$ ) and tended ( $P = 0.09$ ) to graze alfalfa 31% sooner, compared to the INRYE. The ELDIV lambs had a shorter latency to graze alfalfa (-54%) and red clover (-48%) compared with the ELRYE lambs ( $P < 0.05$ ).

## 8.5 Discussion

We accept our hypotheses and to our knowledge are the first to test these hypotheses in a manner that isolates *in utero* and early life effects of diverse and ryegrass dietary exposure in a grazing setting. The results indicate that there is *in utero* and early life transmission of dietary preference and that such differences are more pronounced in lambs with both *in utero* and early-life exposure to the given diets.

*In utero* and early life exposure to ryegrass (INRYE and ELRYE lambs) increased animal's preference towards that species during the preference tests. This is seen in the greater percentage of

time grazing ryegrass, more grazing bouts, and longer bout duration on ryegrass. A similar result was reported for lambs exposed to diverse pastures after exposure (*in utero*–weaning) to a ryegrass diet (control) and supplemented with a seaweed extract; they spent longer grazing ryegrass compared with lambs that were supplemented with an extract comprised of a range of terrestrial plants (Beck et al., 2021). This suggests preference towards ryegrass is the result of neophobia towards novel species and that grazing familiar foods with known post-ingestive feedback allowed them to ingest more feed while intermittently sampling unfamiliar forage species. Other evidence for the diverse treatments (INDIV and ELDIV) showed reduced neophobia towards the non-ryegrass species is seen in reduced latency to graze different species. Moreover, lambs in the diverse treatments spent a greater proportion of their time grazing some of the other species (e.g., plantain and alfalfa). These results provide evidence that *in utero* exposure to diverse diets can increase preference towards given species when re-encountered later in life. While other studies such as Simitzis et al. (2008) and Wiedmeier et al. (2012) have shown an *in utero* effect on preference when animals are exposed to a single novel feed, this paper is the first to evaluate how early-life and *in utero* exposure to five different forages affects forage preference of lambs later in life. For example Simitzis et al. (2008) offered pregnant ewes a plain concentrate-based diet or the concentrate infused with oregano oil, and found lambs with *in utero* oregano exposure had a greater preference towards the oregano feed compared with lambs fed a plain diet during preference testing. Another example of strong effects of *in utero* exposure to diets on early life intake of foods was reported by Wiedmeier et al. (2012), who saw increased intake and digestibility of a high-fiber diet by calves that had *in utero* exposure to the high fiber diet. The greater preference for ryegrass by the INRYE and ELRYE and greater latency to graze alternate species by the RYE treatments compared with diverse exposure is evidence for the potential of *in utero* and early life transmission to reduce the negative implications (e.g. overgrazing, soil compaction, underutilization of feeds within feed budgets, and decreased productivity) of neophobic foraging behaviors.

Preference towards ryegrass was greater for lambs that had both *in utero* and early-life exposure to ryegrass-only compared with the diverse diet, indicating that dietary preference is formulated both *in utero* and during early life exposure. Preference towards ryegrass appeared to get stronger with exposure early in life in addition to *in utero*. This is perhaps due to the continued development and maturation of the taste senses in postnatal lambs. For example, Mistretta and Bradley (1983) found that the sensitivity of tympanic chords (nerve involved in taste) to NaCl and LiCl increases progressively in both pre- and post-natal development. Another study by Mistretta et al. (1988) identified other taste development stages, such as differences in the number of fungiform papillae on the tongue with developmental stage, number of taste buds, and number of taste buds per papillae. More matured development of the taste system or perhaps a greater weight of

importance of early life taste experiences may allow for stronger associations of ingestive properties with post-ingestive feedback, forming longer lasting recognition of feeds. Other differences in how dietary exposure events are weighted and can impact preference in later life can be seen by lambs prioritizing social learning from their dam compared with that from peers (Thorhallsdottir et al., 1990). Another mechanism that could have resulted in these changes in preference that we observed is epigenetic changes. Epigenetic changes are any process that alters gene activity without changing the primary DNA sequence (Welch et al., 2012). Diet, environmental, maternal nutrition, maternal behavior, exposure to toxicants, have all been demonstrated to effect epigenetic profile of animals (Welch et al., 2012). Thereby the oxidative stress experienced by the pregnant dams, different diets, and length of diet exposure could have affected the epigenetics of the lambs and thereby their partial preference and neophobic behaviors. Overall, this experiment is the first to suggest, that providing lambs with diverse diet exposure both *in utero* and in early life reduces dietary neophobia compared with lambs born to ewes offered only ryegrass in *in utero* and in early life. These dietary exposure treatment effects appear to have a stronger influence on dietary preference than *in utero* exposure alone.

Lambs from each treatment mixed their diets, but spent the greatest percentage of their grazing time consuming alfalfa, in agreement with other studies which have reported a greater partial preference for alfalfa by sheep (Pain et al., 2010; Beck et al., 2021), cows (Boland et al., 2011b; Boland et al., 2011a), and goats (Thamina et al., 2020). Further, Beck et al. (2021), Pain et al. (2010), and Boland et al. (2011b) all reported this high preference and low neophobia towards alfalfa from naïve animals, as seen within the present study. Despite this partial preference towards alfalfa and the ability to consume a monotony of alfalfa, animals still choose to compose mixed diets. A similar phenomenon, of selecting a diverse diet, has been described in a number of studies and has been linked to improved welfare (e.g. lowered blood cortisol) (Catanese et al., 2013; Villalba et al., 2015; Beck et al., 2021; Garrett et al., Unpublished), suggesting that a mixture is preferred over consuming a singular familiar food. The provision of choice from a range of familiar foods may enable animals to negate aversions that can occur if a singular food is repeatedly presented, resulting in a diverse diet being preferred by animals (Provenza, 1996).

The strongest evidence for alterations in preference through early life experience is for ryegrass treatments. It is difficult to ascertain the exact diet of the ewes offered the diverse diet and how this impacted preference [through pregnancy and in early life (transmitting experience through milk and social interactions)] as no data were obtained on the preference and grazing patterns of the diverse fed ewes during pregnancy, although the plants available were the same as those used within the preference testing. Future research following the preference of each dam both while they are pregnant and between lambing and weaning could provide further information on how closely the

preference of diverse born and raised lambs matches to their dams. The current results suggest that animals exposed to feeds *in utero* or in early life may have reduced neophobia and thereby improve dry matter intake (DMI) and performance when they encounter those feeds later in life. Further, research into the effect of *in utero* and early life experience to diverse diets on DMI, performance, and thereby economic gain is required. Studies by Wiedmeier et al. (2012) and Wiedmeier et al. (2002) reported that cows exposed to a feed with low digestibility *in utero* or in early life had increased intake of that low-digestibility feed later in life, and had greater weight, body condition score, and milk production compared to animals without early life exposure to such diets, a promising indicator that exposure to diverse diets during this critical period could also yield production gains. Reduction in neophobic behaviors, not only enhance animal well-being by minimizing the stress experienced (Monestier et al., 2017), but could also provide ecosystem services by increasing or maintaining botanical diversity, reducing the over-grazing of familiar feeds, and reduce the resulting damage (e.g., pugging) to areas containing familiar feeds (Launchbaugh and Howery, 2005; Beck and Gregorini, 2020; Beck and Gregorini, 2021). Further, reduced neophobia could improve the utilization of strategically incorporated plants and all feeds included in the feed budget. In addition incorporation of a greater range of plant species where animals “mix the best with the rest” can provide other benefits such as reducing wildfire, conserve and enhance biodiversity, and protect habitats (Meuret and Provenza, 2015a; Meuret and Provenza, 2015b). By utilizing and designing future grazing management alongside future system design to include a greater range of different forage species for enhanced diversity of functional groups with beneficial (e.g., anthelmintic, environmental protective, enhanced production) properties and reduce the requirement for laborious and costly intervention practices (e.g., drenching for parasites) (Meuret and Provenza, 2015a; Meuret and Provenza, 2015b; Beck and Gregorini, 2021).

## 8.6 Conclusions

Forage neophobia can be reduced by *in utero* and early life dietary exposure, with this reduction enhanced both *in utero* and early life experience. In addition, given the choice animals prefer diverse diets over familiar single forage diets. Moreover, reductions of neophobic behaviors as a result of *in utero* and early life exposure could increase intake and performance later in life. Future research following the preference of each dam, both while they are pregnant and between lambing and weaning, could provide further information on how closely the preference of lambs born to and raised by the diverse dams matches their dams' preference.

**Table 8.1** Botanical composition of forage species allocated that were sown into spatially separated strips and provided to lambs for preference testing

Species	Herbage					SEM <sup>1</sup>
	Chicory	Red clover	Alfalfa	Plantain	Ryegrass	
Herbage mass, kg DM/ ha	2285	3138	3349	2537	3524	134
Leaf, % DM	60.23	60.17	76.26	58.70	67.26	4.22
Reproductive, % DM	28.94	23.20	-	26.79	6.33	3.90
Weed, % DM	0.88	5.45	9.19	0.53	0.99	1.86
Dead, % DM	9.95	11.18	14.09	13.99	25.41	2.39
Extended shoot leaf height (cm)	19.3	17.0	26.8	17.7	19.1	1.9
Reproductive stem extended height (cm)	25.4	20.7	-	25.6	24.4	1.9

<sup>1</sup> SEM = standard error of the mean

**Table 8.2** Primary chemical composition of forage species allocated that were sown into spatially separated strips and provided to lambs for preference testing.<sup>1</sup>

Item <sup>2</sup>	Herbage					SEM <sup>3</sup>
	Chicory	Red Clover	Alfalfa	Plantain	Ryegrass	
ME, MJ/kg DM	10.91	10.25	10.54	10.15	11.57	0.21
DM, % as-fed	10.09	16.44	13.72	16.26	22.99	0.90
DMD, % DM	72.98	68.96	69.23	66.00	73.76	1.19
CP, % DM	14.70	20.38	24.75	10.30	12.10	0.58
NDF, % DM	24.39	31.16	30.81	35.12	48.18	1.29
ADF, % DM	25.89	27.12	28.24	28.57	27.22	0.76
WSC, % DM	11.06	10.63	8.66	15.04	21.23	1.19

<sup>1</sup> Nutritive quality of the forage species was not different by treatments ( $P \geq 0.14$ )

<sup>2</sup> ME = Metabolizable energy; DM = Dry matter; DMD, DM digestibility predicted by Near infrared spectroscopy; OM = organic matter; OMD = OM digestibility; WSC = water soluble carbohydrates; NDF = neutral detergent fiber; ADF = acid detergent fiber; CP = crude protein

<sup>3</sup>SEM = standard error of the mean

**Table 8.3** Proportion of time spent conducting a behavior and the number and duration of grazing bouts of lambs undergoing preference testing. Lamb treatments were exposure to the diverse plant species in the spatially separated strips only *in utero* (INDIV), or *in utero* and in early life alongside their dam (ELDIV), or were exposed to ryegrass *in utero* (INRYE) or were exposed to ryegrass *in utero* and in early life alongside their dam (ELRYE).

Behavior	TRT				P-value <sup>1</sup>		
	INDIV	INRYE	ELDIV	ELRYE	IN	EL	IN vs. EL
Time spent, %							
Grazing	48.66 ± 2.06	49.33 ± 2.12	50.78 ± 2.24	50.18 ± 2.16	0.73	0.76	0.28
Idle	36.65 ± 3.13	36.53 ± 3.21	34.71 ± 2.91	34.42 ± 2.81	0.96	0.90	0.21
Ruminating	10.61 ± 1.92	10.48 ± 1.84	10.66 ± 1.92	11.44 ± 2.17	0.90	0.45	0.51
Grazing bout, n	44.39 ± 2.01	40.22 ± 1.74	44.48 ± 2.05	44.23 ± 2.00	0.04	0.91	0.13
Bout duration, min	9.88 ± 0.34	10.89 ± 0.41	10.08 ± 0.36	10.03 ± 0.35	0.02	0.92	0.27

<sup>1</sup> P-value of fixed effects; TRT<sup>1</sup> = Treatment; <sup>2</sup> IN = Contrast between the two treatments (INDIV vs. INRYE) that only had *in utero* exposure to the specified dietary treatments; EL = contrast between the two treatments (ELDIV vs. ELRYE) that had *in utero* and early life exposure alongside dams to the specified dietary treatments; IN vs. EL = contrast between the treatments that had *in utero* (IN) dietary exposure and those that had *in utero* and early life exposure alongside the dams



**Table 8.4** Proportion of time spent grazing chicory, alfalfa, plantain, red clover, and ryegrass of lambs undergoing preference testing. Lamb treatments were exposure to the diverse plant species in the spatially separated strips only *in utero* (INDIV), or *in utero* and in early life alongside their dam (ELDIV), or were exposed to ryegrass *in utero* (INRYE) or were exposed to ryegrass *in utero* and in early life alongside their dam (ELRYE)

Behavior	TRT				Contrasts <i>P</i> -value <sup>1</sup>		
	INDIV	INRYE	ELDIV	ELRYE	IN	EL	IN vs. EL
Grazing, % time grazing in							
Chicory	10.72 ± 1.70	9.33 ± 1.37	7.78 ± 0.99	7.66 ± 0.99	0.40	0.91	0.03
Alfalfa	46.28 ± 4.02	38.79 ± 3.12	44.19 ± 3.82	40.66 ± 3.25	0.03	0.30	0.96
Plantain	12.16 ± 2.13	10.58 ± 1.64	17.77 ± 4.67	7.71 ± 0.92	0.36	0.02	0.45
Red clover	15.87 ± 1.79	19.38 ± 2.46	15.27 ± 1.68	14.03 ± 1.44	0.06	0.39	0.02
Ryegrass	12.32 ± 1.84	15.55 ± 2.69	16.51 ± 3.04	26.53 ± 7.06	0.10	0.05	0.02

<sup>1</sup> *P*-value of fixed effects; TRT<sup>1</sup> = Treatment; <sup>2</sup> IN = Contrast between the two treatments (INDIV vs. INRYE) that only had *in utero* exposure to the specified dietary treatments; EL = contrast between the two treatments (ELDIV vs. ELRYE) that had *in utero* and early life exposure alongside dams to the specified dietary treatments; IN vs. EL = contrast between the treatments that had *in utero* (IN) dietary exposure and those that had *in utero* and early life exposure alongside the dams

**Table 8.5** Grazing bout information (duration and number) by species: chicory, alfalfa, plantain, red clover, and ryegrass, of lambs undergoing preference testing. Lamb treatments were exposure to the diverse plant species in the spatially separated strips only *in utero* (INDIV), or *in utero* and in early life alongside their dam (ELDIV), or were exposed to ryegrass *in utero* (INRYE) or were exposed to ryegrass *in utero* and in early life alongside their dam (ELRYE).

Behavior	TRT				Contrasts <i>P</i> -value <sup>1</sup>		
	INDIV	INRYE	ELDIV	ELRYE	IN	EL	In vs. EL
Bout number, count							
Chicory	6.71 ± 0.95	5.56 ± 0.70	5.26 ± 0.63	5.01 ± 0.59	0.19	0.69	0.07
Alfalfa	17.57 ± 0.96	14.39 ± 0.72	17.31 ± 0.96	15.42 ± 0.78	<0.01	0.05	0.54
Plantain	6.22 ± 0.92	5.44 ± 0.73	9.63 ± 2.24	4.97 ± 0.60	0.32	0.02	0.10
Red clover	8.08 ± 0.88	7.15 ± 0.74	6.94 ± 0.71	6.87 ± 0.67	0.24	0.92	0.17
Ryegrass	5.94 ± 0.77	6.58 ± 0.91	7.20 ± 1.08	10.56 ± 2.16	0.33	0.02	<0.01
Bout duration, min							
Chicory	7.23 ± 0.55	7.43 ± 0.58	6.18 ± 0.42	6.87 ± 0.52	0.74	0.19	0.05
Alfalfa	11.53 ± 0.63	11.08 ± 0.63	12.06 ± 0.72	11.64 ± 0.66	0.57	0.64	0.32
Plantain	7.81 ± 0.65	8.90 ± 0.84	7.58 ± 0.64	6.64 ± 0.48	0.17	0.14	0.01
Red clover	8.27 ± 0.71	11.50 ± 1.27	10.30 ± 1.07	9.26 ± 0.85	<0.01	0.24	0.87
Ryegrass	7.87 ± 0.68	9.52 ± 0.96	9.13 ± 0.88	11.25 ± 1.26	0.06	0.05	0.03

<sup>1</sup> IN = Contrast between the two treatments (INDIV vs. INRYE) that only had *in utero* exposure to the specified dietary treatments; EL = contrast between the two treatments (ELDIV vs. ELRYE) that had *in utero* and early life exposure alongside dams to the specified dietary treatments; IN vs. EL = contrast between the treatments that had *in utero* (IN) dietary exposure and those that had *in utero* and early life exposure alongside the dams.

<sup>2</sup> SEM = standard error of the mean

**Table 8.6** Latency until grazing in chicory, alfalfa, plantain, red clover, and ryegrass of lambs undergoing preference testing. Lamb treatments were exposure to the diverse plant species in the spatially separated strips only *in utero* (INDIV), or *in utero* and in early life alongside their dam (ELDIV), or were exposed to ryegrass *in utero* (INRYE) or were exposed to ryegrass *in utero* and in early life alongside their dam (ELRYE).

Behavior	TRT				Contrasts <i>P</i> -value <sup>1</sup>		
	INDIV	INRYE	ELDIV	ELRYE	IN	EL	In vs. EL
Latency to graze, min							
Chicory	130.3 ± 17.83	230.4 ± 39.78	212.7 ± 35.84	167.7 ± 27.04	0.02	0.28	0.73
Alfalfa	56.7 ± 10.94	82.9 ± 20.24	35.7 ± 5.72	77.5 ± 17.55	0.09	<0.01	0.13
Plantain	94.8 ± 46.84	96.3 ± 48.31	84.4 ± 37.40	102.4 ± 54.38	0.88	0.37	0.77
Red clover	102.7 ± 24.17	137.7 ± 40.17	65.7 ± 13.68	126.7 ± 35.21	0.25	0.05	0.22
Ryegrass	94.4 ± 44.82	91.3 ± 41.99	86.3 ± 37.52	80.5 ± 32.85	0.72	0.51	0.34

<sup>1</sup> IN = Contrast between the two treatments (INDIV vs. INRYE) that only had *in utero* exposure to the specified dietary treatments; EL = contrast between the two treatments (ELDIV vs. ELRYE) that had *in utero* and early life exposure alongside dams to the specified dietary treatments; IN vs. EL = contrast between the treatments that had *in utero* (IN) dietary exposure and those that had *in utero* and early life exposure alongside the dams.

<sup>2</sup> SEM = standard error of the mean

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## Chapter 9

### General Discussion

This Ph.D. thesis aimed to determine the effects of altering the functionality of diverse diets (through context, species abundance, species distribution, and temporal availability) on intake, production, welfare, and the environmental impact of farming practices compared with those grazing a monotonous diet. The functionally diverse diets included different combinations, sequences, and abundances of perennial ryegrass (*Lolium perenne* L.), chicory (*Cichorium intybus* L.), alfalfa (*Medicago sativa* L.), red clover (*Trifolium pratense* L.), and plantain (*Plantago lanceolata* L.). Diets allocated in monotony included perennial ryegrass, alfalfa, or a homogenized mixture of equal parts dry matter of ryegrass, chicory, alfalfa, and plantain. I hypothesized that providing a functionally diverse diet as opposed to a monotonous diet would: 1) alter fermentation patterns, increase production, and reduce negative environmental impacts, 2) enhance animal welfare, and 3) alter foraging behavior through *in utero* and early life experience. I also hypothesized that providing functional diversity would impact 1-3) to a greater extent than non- or low-functionally diverse diets. The subsequent sections outline a brief introduction into each hypothesis and the rationale for testing, the subsidiary hypothesizes, and then discuss supporting evidence from relevant Chapters, as well as considering these results alongside other literature, and key areas of future research this thesis has highlighted.

The results comparing functionally diverse diets to monotony are context specific. As a result, the hypotheses for this thesis are accepted or otherwise based on the contexts that they were explored within the thesis. The diverse diets examined within the context of this Ph.D. research provide promising results for use of the principles of functionally diverse diets in other contexts.

#### 9.1 Hypothesis One

Every level of livestock production systems, from animal health, growth, welfare, production, the environmental footprint of practices to financial viability are affected by the nutrition and management of animals (Vasta et al., 2008; Vasta and Luciano, 2011; Gerber et al., 2013; Makkar and Ankers, 2014; Vazirigohar et al., 2014; Makkar, 2016). However, within intensive pastoral systems often one feed type can meet the average nutritional requirement of the herd. An example of one such diet is ryegrass (*Lolium perenne* L.) and white clover (*Trifolium repens*) which are commonly fed year round in countries such as New Zealand, as they provide a quality and high yielding diet under a range of temperate conditions and management styles (Carlson et al., 1996; Delagarde et al.,

2000). Such diets may meet the nutrient requirements of the average animal, however many animals fall outside of this average due to the broad ranging individuality within herds as a result of unique genetics, morphology, physiology, sex, developmental stage, nutritional state, and experience (Provenza et al., 1996; Scott and Provenza, 1999; Manteca et al., 2008). Such diets grossly oversupply some nutrients (e.g. CP) and thereby result in low nutrient use efficiency. Inefficiencies in nutrient utilization can represent a financial cost, through additional feed supply, reduced animal production, and increased negative environmental impact by excess of nutrient excretion to the environment (e.g. urinary N). Further, such excess nutrient excretion have even been associated with aversive affects for human health. For example, excess levels of nitrates in water supplies have been linked to colorectal cancer (Schullehner et al., 2018) and blue baby syndrome (Fossen Johnson, 2019). Such diets are often fed in monotony (repetitively), which may exacerbate inefficiencies. As such, interest in finding alternative diets with greater nutrient use efficiency has grown, particularly those that incorporate a diverse array of feed components (Atwood et al., 2006; Distel et al., 2007; Catanese et al., 2013; Jensen et al., 2013; Villalba et al., 2015b). However, much of this research has been conducted using grains, concentrates, silages, or mixed swards with no- or low- functionality, or feds that provide animals no choice or divergence in chemical or oro-sensorial experience. Thereby, the first hypothesis of this thesis was broken into three subsidiary hypotheses that a functional fresh forage diverse diet as opposed to a dietary monotony would alter: a) rumen fermentation patterns, b) increase production and, c) reduce the negative environmental impact.

### **9.1.1 Rumen Fermentation**

Part a) of hypothesis one is that dietary diversity will alter rumen fermentation patterns. This hypothesis was tested in experiments presented in Chapters 3, 5 and 6. Within Chapter 3 different proportions of ryegrass to chicory, alfalfa, and plantain, or a mixture (equal parts DM of chicory, alfalfa, and plantain) were compared using an *in vitro* ANKOM<sup>RF</sup> Gas Production System. To my knowledge, this is the first experiment to compare a diverse forage diet to a single component *in vitro*. The proportion of chicory, plantain, or the mixture increased 24 h gas production and branch-chained volatile fatty acid production, while reducing ammonia (NH<sub>3</sub>) production. One of the *in vivo* experiments (Chapter 6) showed a 75% reduction in ruminal NH<sub>3</sub> concentrations of animals grazing a diverse diet, while the other *in vivo* experiments that examined ruminal parameters (Chapter 4 and 5) showed no difference. The *in vitro* and Chapter 6 result of reduced ruminal NH<sub>3</sub> from the diverse treatment also suggest that functionally diverse diets reduce the negative environmental impact of farming practices (part c of the first hypothesis). Ruminal NH<sub>3</sub> is a by-product of ruminal protein breakdown that is absorbed and transported from the rumen to the liver, where it is readily converted to urea and excreted in the urine (Attwood et al., 1998). Other rumen characteristics differed between treatments in a number of the experiments, including a reduced acetate to

propionate ratio of diverse diet treatments, seen as a tendency in Chapter 4 and a difference in the AM for the varied functional diversity treatment in Chapter 6. Lower acetate to propionate ratios are indicative of reduced rumen methanogenesis due to stoichiometric ratios of less acetate (which produces free hydrogen during its formation that can be utilized during methanogenesis) and more propionate (which acts as a hydrogen sink) (Rivero et al., 2020), thereby providing further proof to part c) of hypothesis one regarding reduced environmental impact of functional diversity. The diverse treatments also had a greater proportion of the total VFA profile comprised of butyrate in Chapter 5. The proportion of total VFA comprised of butyrate was numerically greater for the diverse diet in Chapter 4, and butyrate values increased with increasing incorporation of chicory and the diverse mixture in Chapter 3. This rumen fermentation difference has implications for another part of the hypothesis, part b) regarding animal production, as butyrate is the primary energy source for epithelial cells and stimulates their proliferation and thereby feed utilization (Miguel et al., 2019). Other differences in rumen characteristics existed between diverse and monotonous treatments, however, these differences were not as consistent among experiments. The chemical composition of the diverse and monotonous diets differed between experiments and inconsistencies in rumen parameters could have been due to differences in the chemical composition of the forages. Further, some of the diverse diets offered low to no functionality as they enforced set ratios of the diet components thereby rumen characteristics may have been more similar to the monotonous diets than those offered free choice. For example, there was a 75% reduction in ruminal  $\text{NH}_3$  for the varied free choice diet lambs compared to the monotonous alfalfa treatment in Chapter 6, while there was no difference in rumen  $\text{NH}_3$  between the set ratio diverse diet and ryegrass diet in Chapter 5. Based on these results, it is apparent that a functionally diverse diet can alter fermentation patterns by increasing rate of fermentation and altering fermentation characteristics compared to a monotonous diet. Although, the results from Chapter 4 presented no difference in ruminal parameters from a monotonous mixture and a restricted choice diversity of the same composition, they indicate there is less benefit in providing choice if the rations are offered in restricted proportions.

Although this thesis provided some of the only rumen data available for diverse diets, the data both *in vitro* and *in vivo* were limited by the sampling frequency to evaluate rumen fermentation patterns. Arguably, future research *in vivo* with animals fitted with a rumen cannula or *in vitro* using a continuous system that allows substrate incorporation and sample extraction through time could enhance understanding of both varied and diverse diets effect on rumen fermentation patterns and function. Further, analysis of the rumen microbiome would also increase understanding of diverse diets on rumen characteristics. Information on rumen microbiome could provide insights into animals microbiota-gut brain axis. Such information collected alongside welfare data could link

rumen microbiota with animal welfare, as work has been done in humans, where the gut microbiome has been linked with mood, behavior, and welfare (see Cryan and Dinan, 2012).

### 9.1.2 Animal Production

Expanding on the large body of literature comparing a range of concentrates or mixed diverse swards with low functionality to a total mixed ration or single forage diet (Distel et al., 2007; Golding et al., 2011; Villalba et al., 2011; Al-Marashdeh et al., 2020), Part b of the first hypothesis was that a functional fresh forage diverse diet would increase production compared with dietary monotony. This hypothesis was explored in the experiments in Chapters 3, 4, 5, 6, and 7.

In Chapter 3, the *in vitro* experiments showed increased gas production after 24 hrs, reduced ruminal NH<sub>3</sub> and branched-chain VFAs, suggesting a greater energy supply from the rumen to the host animal and an increase in microbial protein synthesis (Miller et al., 2001; Krause et al., 2002; Vibart et al., 2009; Totty et al., 2013). Increasing microbial protein flow to the duodenum, which is the greatest contributor of metabolizable protein for ruminants (Clark et al., 1992; Firkins, 1996), suggests benefits to animal production *in vivo*. Such inferences were supported by the results reported in Chapters 4, 5, 6, and 7. In Chapter 4, the animals in the diverse treatment (approximately equal parts DM chicory, plantain, alfalfa, and ryegrass) consumed 48% more dry matter and gained 92% more live weight per day compared with lambs on the monotonous ryegrass diet. Further, the DMI of the diverse treatment was more consistent across days (lower DMI CV%). Lower DMI CV% has been correlated with improved ADG in a number of studies (Allison, 1985; Galyean et al., 1992; Horn et al., 2005; Williams et al., 2018). The benefits in production were likely the result of nutritional differences. Chapter 5 compared intake, ruminal, and blood parameters of a monotonous ryegrass treatment, a monotonous mixture (approximately equal parts DM chicory, plantain, alfalfa, and ryegrass), and a variation in the temporal availability of the same diet components within the mixture. The DMI of lambs on the varied functional diversity treatments was 20 and 10% greater and the day-to-day coefficient of variation of DMI was 29 and 23% lower compared to the monotonous ryegrass and mix treatments, respectively. Collectively, the greater DMI and reduced DMI CV% alongside the greater ruminal butyrate of the varied diet lambs, is indicative of greater epithelial cell proliferation and nutrient use efficiency (Miguel et al., 2019), which suggests that ADG would also be greater than the monotonous diets. Chapter 6 compared a monotonous diet of alfalfa, known and utilized within NZ and other pasture based systems as one of the most high-performing and quality finishing diets for lambs, with a functionally diverse diet (free choice from spatially separated chicory, plantain, alfalfa, and ryegrass), and a varied diet (free choice from ryegrass and plantain in the morning, and alfalfa and chicory in the evening). At a similar forage DMI, the ADG of the diverse and varied diet lambs was 30 and 67% greater, respectively, than that of the alfalfa lambs (227 g/d), and

the varied diet lambs ADG was 28% greater than the diverse lambs. Interestingly, the DMI and the primary composition of the diets consumed by the varied and diverse lambs were not different, indicating that differences in functionality, secondary chemistry, or welfare may have contributed to the differences in ADG. Chapters 5 and 6 both provide evidence not only to diverse diets increasing animal performance, but also that diets providing additional functionality through the temporal sequence can have a greater impact than free choice alone, as hypothesized in the literature review (Chapter 2).

Future research feeding the diets from Chapter 6 for a longer period is warranted. Unfortunately, the Covid-19 pandemic resulted in a nationwide lockdown which halted this experiment 20 days early. Nevertheless, the animals were well adapted to the alfalfa diet, as they were grazing it prior to the experiment and the diverse and varied diet animals adapted quickly to their diets, evidenced by their DMI CV being equivalent or lower than the alfalfa diet. Chapter 7 provided additional evidence for production increments on functionally diverse diets by comparing two groups of ewes fed a ryegrass only diet or a diverse diet of spatially separated strips of chicory, plantain, alfalfa, red clover, and ryegrass in the last third of gestation. Ewes in the diverse treatment birthed lambs that were 9% heavier than the ryegrass treatment, despite the diets DM and ME content being the same. Greater lamb birth weight can be indicative of increased production, as that has been associated with decreased mortality (Morel et al., 2009).

Collectively, these results support the acceptance of hypothesis one b), in that a functionally diverse fresh forage diet can increase animal production and these effects are more marked when diversity is provided in a more functional manner (i.e. spatially separated or varied temporal allocation). While, this thesis provided evidence that functional diversity improves production, further research is required to explore the quantities, spatial arrangements, and temporal distributions (sequences) that elicit the most promising effect within a range of contexts (i.e. different animal breeds, environments, and feed types). The next step would be to conduct both an indoor and outdoor trial where equal proportions of herbage species are offered a mixture and performance is compared with animals offered the same species in the same ratios, but spatially separated to provide more evidence that how the diets offered matters. Although this PhD research identified that it is more than the primary chemistry that effects production, much of this research occurred within controlled settings, and further research is required to evaluate spatial and temporal arrangements within practical grazing settings to translate this research into manageable on farm practices. Issues that arose during the grazing trial (Chapter 7 and 8) involved management of grazing of pastures with different defoliation requirements. Future research should explore how planting adjacent plant combinations that require similar management could provide a diverse diet within paddock and a varied diet between paddocks, allowing for the practical translation of this research

into grazing settings. Such research should occur with a range of animal species to determine what spatial and temporal arrangements of feeds provide dietary diversity in a functional manner as outlined within the literature review.

### **9.1.3 Environment**

The final part of hypothesis one c) was that a functionally diverse diet as opposed to dietary monotony would reduce the negative environmental impact of grazing. This hypothesis was tested in the experiments presented in Chapters 3, 4, 5, and 6. Experiments described in Chapters 3, 5, and 6 showed differences in rumen fermentation characteristics namely the increased gas production, reduced branch-chained volatile fatty acid production, reduced acetate to propionate ratio, and reduced  $\text{NH}_3$  production for the diverse treatments. These results were indicative of increased nutrient supply to the rumen microbes, increased performance of the host animal, reduced methane, and reduced ruminal  $\text{NH}_3$  from the diverse treatments compared with the monotonous treatments. The increases in production discussed in section 1.1.2. are suggestive of reduced days to slaughter and reductions in emission intensity (pollutant per kg of product) (Capper et al., 2009; Beck and Gregorini, 2021). For example, extrapolating data from diets of differing functionality from Chapter 6, lambs (starting live weight ~34 kg) could have been finished (~40 kg) on the alfalfa only diet after 26 days, a free choice functional diverse diet after 20 days, and a temporally varied functional diverse diet after 16 days, indicating that days to slaughter could be reduced by 23-38% by a diverse diet. Chapter 4 reported that lambs had a 30% reduction in urinary N excretion from the diverse treatments (as suggested by the reduced ruminal  $\text{NH}_3$  in the fermentation section 1.1.1.) compared with the monotonous ryegrass treatment. As there is a known curvilinear relationship between N loading at the urine patch level and N leaching (Di and Cameron, 2007; Li et al., 2012), these results suggest that the magnitude of difference between the monotonous ryegrass and diverse treatment could be 30%, or even greater, for nitrate leaching. This supports the hypothesis that functionally diverse forage diets can reduce the negative environmental impact of animals relative to those grazing a monotonous single forage diet, while maintaining or increasing animal performance. The greater magnitude of production benefits for the functionally diverse diets provides some evidence for reduced environmental impact from a functionally diverse diet compared with a monotonous or non-functional diversity, however as this was the only environmentally based difference reported, it is not enough to reach a conclusion for this part of the hypothesis. Future research with more frequent urinary N measurements and methane emissions could provide further insight into the environmentally protective potential of diverse diets. Further, the cause for single forage diets high urinary N excretion has been hypothesized to be a product of incidental augmentation, where CP intake is excessive to meet energy and other nutrient demands, however future research is required to explore this as a cause.

## 9.2 Hypothesis Two

Consumers are placing increasing pressure on livestock-production systems to enhance animal welfare (Gregorini et al., 2017). Such pressure has stemmed from consideration of animal welfare beyond the five freedoms: Freedom from (1) thirst, hunger, and malnutrition, (2) discomfort, (3) pain, injury, and disease, (4) to express normal behavior, and (5) fear and distress (Council, 1993; Webster, 2016), to a 'life worth living' that encompasses eudaimonic wellbeing (pursuit of purpose) (Mellor, 2016; Beck and Gregorini, 2020). By nature, dietary monotony violates four (1-4) of the freedoms. While diets allocated to meet the requirements of the average animal may not provide a 'life worth living' and may also impair four of the five freedoms (1-4), as they can provide ill-fitting diets to meet nutritional needs, provide nutritional discomfort, impair health, and prevent animals from conducting normal preference and selective behaviors. Moreover, monotonously fed diets provide invariant sensorial experience that can induce boredom (Burn, 2017; Villalba and Manteca, 2019), which in concentrate feeding based settings reduces welfare (Catanese et al., 2013). Exploration of the effects of dietary monotony and diversity on animal welfare has been limited. Therefore, the second hypothesis was that a functionally diverse fresh forage diet would enhance animal welfare relative to dietary monotony.

Evidence for improved welfare is suggested by the increased animal performance from the diverse diets compared to the monotonous diets, and even greater production of the functionally diverse diets compared to the non- or less-functionally diverse diets (see section 1.1.2). Increased performance can be considered as a proxy of welfare when animals gain or produce at a rate suitable to their context (Roche et al., 2009; Barrell, 2019). Greater performance has been correlated with reduced day-to-day coefficient of variation of DMI in a number of other studies (Allison, 1985; Galyean et al., 1992; Horn et al., 2005; Williams et al., 2018). Greater CV indicates cyclic patterns of intake due to transient food aversions (Provenza, 1996). I also found reduced day-to-day coefficient of variation of DMI from functionally diverse diets compared to non- or low functionally diverse diets and monotonous diets reported in Chapters 4, 5, and 6. Further, reduced day-to-day coefficient of variation of DMI has been linked to reduced health incidents (McGuffey et al., 1997) and consequently can be indicative of enhanced welfare. Elevated ruminal NH<sub>3</sub> reported in Chapter 3 (In vitro) and in Chapter 6 (In vivo) from the monotony treatments could also be indicative of reduced welfare as excessive levels of dietary N can have detrimental effects on animal health (e.g. impaired fertility) (Pacheco and Waghorn, 2008) and they condition food aversions (Provenza, 1996). Chapters 4, 5, and 6, which examined a range of functionally diverse diets and a range of monotonous diets, reported that animals supplied functionally diverse diets spent a lower proportion of their day or part of their day (morning or evening) conducting stereotypic behaviors or conducted fewer bouts of stereotypic behaviors than either the monotonous or no to low- functionality treatments.

Stereotypic behaviors are repetitive behaviors with no apparent function although some speculate they help animals cope with stress in their environment (Broom, 1991; Catanese et al., 2013). The general consensus is that they are indicative of poor welfare.

Plasma total antioxidant status (TAS) depicts the total balance between reactive oxygen species (ROS) and antioxidants (exogenous defense against oxidative damage), and therefore is an overarching representative of the antioxidant-to-oxidant balance. Moreover, TAS can be indicative of reduced oxidative stress or increased capacity to cope with oxidative stress, and can be a sign of improved internal state and hedonic well-being of ruminants (Beck and Gregorini, 2020). Glutathione peroxidase (GPx), another marker of oxidative stress, can indicate the presence of a stressor (Bernabucci et al., 2002; Beck et al., 2021b) or improved antioxidant status due to greater intake of precursor materials (e.g. selenium) (Gerloff, 1992), thereby it is best interpreted alongside other markers. Elevated non-esterified fatty acids (NEFA) indicate greater mobilization of fat, which has been associated with increased oxidative stress (Sordillo and Aitken, 2009; Sordillo and Mavangira, 2014; Li et al., 2016). The effects of functional diversity on the TAS and GPx were mixed between experiments. Chapter 4 reported no differences between treatments. Chapter 5 saw the mixed no-low functionality diet having a lower TAS than the monotonous ryegrass and functionally diverse treatments, and no differences in GPx. Chapter 6 blood parameters were considered alongside the stereotypic behavior and intake data to interpret their collective meaning. I concluded lambs in the monotonous alfalfa treatment exhibited greater oxidative stress (lower afternoon TAS, reduced performance, tendency for a greater DMI CV%, and an increased number of stereotypic behavior bouts) relative to the free choice and varied functional diversity treatments. Finally Chapter 7, examined the TAS, GPx, and NEFA concentration in plasma and blood from ewes grazing a functionally diverse or ryegrass only diet 24 hrs after lambing. The diverse ewes had a greater antioxidant status and reduced metabolic stress as evidenced by the greater GPx and TAS, and lower NEFA. These collective results lead me to accept the hypothesis that a diverse diet can enhance animal welfare.

The most marked differences in welfare with blood parameters to support production, DMI CV, and stereotypic behavior were observed in Chapters 6 and 7 when the diverse and varied treatments were supplied with more functional diversity than the studies reported in other Chapters where ratios of diverse feed components were fixed. These discrepancies between less functionally diverse diets (although still functional) and the more functional diverse diets lead to the acceptance of the hypothesis that a functionally diverse fresh forage diet enhances animal welfare relative to those offered dietary monotony. The evidence presented in this thesis provides valuable information on fresh forage diets to be considered alongside the improvements in welfare outlined by Catanese et al. (2013) using concentrate based diets. However, further research that assesses key indicators of



welfare [stress (e.g. cortisol and fructosamine) or positive welfare or eustress (e.g. serotonin)] is required before this hypothesis can be accepted fully in terms of fresh forages. Future research should also explore if functionally diverse diets allow animals to respond and cope with stressors better than animals offered a single feed diet. Finally, research to explore the effects of high diet richness or level of varied-ness is required to determine if there is a level where too much 'diversity' is detrimental, and thereby becomes non-functional or impairs welfare.

### 9.3 Hypothesis Three

The presentation of 'novel' feeds can induce neophobia (fear of new) where animals avoid consuming unfamiliar feeds, reducing feed intake and subsequently animal performance and economic gain (Launchbaugh et al., 1997). Further, neophobia can cause animals to overgraze familiar feeds thus degrading landscapes, and underutilize feeds accounted for within feed budgets or strategically incorporated into systems for their properties (e.g. medicinal, environmental) (Launchbaugh and Provenza, 1991; Beck et al., 2021a). Neophobic behaviors have been reported to be reduced if the forage has high hedonic value (e.g. induces pleasure); however, plants with high concentrations of PSC with beneficial properties (e.g. medicinal, therapeutic, or environmental) can also impart bitter flavors, thereby have low hedonic value (Ginane et al., 2011; Beck and Gregorini, 2021). At weaning animals are often moved to new foraging environments for finishing or fattening purposes where they encounter feeds that are novel to them, representing a potential economic loss, as decreased feed intake reduces productivity and increases the time it takes to finish them. Thus, the negative effects of neophobia require it to be minimized within controlled grazing systems. Early life exposure through *in utero*, milk ingestion, social interactions, and consumption of feeds influence behavioral patterns and preference in later life (Arnold and Maller, 1977; Beck and Gregorini, 2021). Therefore, the final hypothesis of this thesis was that *in utero* and early life (*in utero* through to weaning) exposure to diverse forage diets would alter grazing behavior and partial preference in later life compared to those on a monotonous diet. A subsidiary hypothesis was that animals offered early life exposure to the diverse diets would exhibit greater differences to the monotonous ryegrass treatments compared to the *in utero* treatment. The final subsidiary hypothesis was that regardless of prior experience, lambs would choose to mix their diet.

This hypothesis was explored in the experiment presented in Chapter 8, where each twin lamb had either *in utero* only or early life (*in utero* through to weaning) exposure to a functionally diverse diet of ryegrass, red clover, plantain, chicory, and alfalfa (INDIV or ELDIV) or a monotonous diet of ryegrass (INRYE or ELRYE). The ELRYE lambs spent more time grazing ryegrass than their counterparts (ELDIV), while for the *in utero* treatments the same treatment effect was only a tendency. The ELRYE lambs achieved a greater grazing time in ryegrass by increasing bout number and bout duration,

while INRYE lambs increased their bout duration within ryegrass. These results indicate that the lambs were displaying neophobic behavior or preferential grazing of that of which was familiar to them, and that this effect was stronger in animals that had a longer early life exposure to the given diet. Further, the INDIV and ELDIV both displayed reduced latency (time to enter a species and graze it) to graze some species compared to the ryegrass treatments. Therefore, I accept the main hypothesis that functional diversity alters grazing behavior and partial preference later in life compared to a monotonous diet. I also accept the hypothesis that lambs with early life exposure to the diverse diets would exhibit greater differences to the monotonous ryegrass only treatments compared to the *in utero* treatment. Finally, I accept that animals, when given choice to comprise their diet of a range of high quality, individual diet components, still prefer to mix their diet regardless of prior experience. This adds to a growing body of literature that supports such a phenomenon and highlights that animals naturally select a diverse diet if afforded the opportunity and that no single food can meet needs for both primary and secondary compounds (Villalba et al., 2015b; Villalba et al., 2015a; Beck et al., 2021a). Further, this evidence provides further information to existing literature, such as Beck et al., 2021a, on the plasticity and ability to pre-program preference and reduce neophobic behaviors negating the associated negative effects. To my knowledge this paper was the first to pinpoint the effect of feeding a diverse forage diet on *in utero* partial preference and neophobia development and compare it to animals with *in utero* and early life exposure to a single forage diet. Future research following the preference of diverse fed dams, both while they are pregnant and between lambing and weaning, could provide further information into how closely the preference of lambs born to and raised by the diverse dams matches their dams. Furthermore, research examining the productivity of such treatments is required to determine the longevity and productive effects of such early life dietary effects.

## 9.4 Summary

In summary, the literature review and results of this thesis added to the body of growing literature on the beneficial effects of dietary diversity compared to single feed or repetitive diets, which address major areas of societal concern, meeting food production demands, improving animal welfare, and reducing the negative environmental impact of livestock farming practices. The literature review introduced a new concept of functional dietary diversity and hypothesized how these principles could be incorporated into the system and attempted to explain some of the variability in results of diverse diets compared with monotonous diets reported to date. Collectively, the results of this thesis suggest that functionally diverse diets can provide benefits above the primary chemistry expected from single forage diet or mixed diverse diet and that use of functionally diverse diets can meet growing consumer demands as hypothesized in the literature review. Although the results from the current thesis were limited in that we were unable to test a monotony

of each plant type offered within the diverse diet as a whole diet, leaving some questions of if the results seen were a result of chemical differences alone. However, none of the single forage diet components provided a chemistry close to the average chemistry of the diverse diet, or were consumed as the whole diet by the diverse animals offered free choice. Further, a mixture would be unable to reliably replicate the given composition of the functionally diverse diets across multiple grazing events. The mechanisms suggested to increase production and reduce environmental impact were greater nutrient use efficiency, the implicit PSC, and increased welfare. This opens up an avenue for future research on functional diversity. The results presented in Chapters 3, 4, 5, 6, and 7 allow the conclusion that functional diversity can improve production while reducing the environmental impact of farming practices compared to monotonous diets. The results reported in Chapters 6 and 7 provided the most marked differences in welfare (blood parameters, production, DMI CV, and stereotypic behavior differences were observed), which occurred when the diverse diet supplied was most functional allowing free choice to comprise diets at will. The results reported in the other Chapters provided mixed effects on welfare, however, if an effect was present it was indicative of improved welfare for animals consuming a diverse diet. The mixed results were presumably a result of the functionality of the diverse diets within these studies being more restricted in terms of supplying set ratios of herbages. Finally, the results presented in Chapter 8 provided evidence that familiarity with diverse diets *in utero* or in early life can reduce neophobic behaviors in later life, which may improve utilization of all allocated feeds (in terms of feed budget and strategic incorporation for medicinal properties) and increase animal production. The experiments in this thesis explored a range of monotonous diets and diets with different functional diversities, which included a range of contexts (times of year, settings, animal ages, and developmental stages) with different spatial (mixes or spatially separated) and temporal arrangements (sequence feedings). These diet ranges determined that diverse diets can improve production, welfare, and environmental impact compared to single forage diets, but these effects are more marked if they are provided in a functional manner. This thesis has provided valuable information for pastoral and otherwise livestock systems, in that it is not just what you feed but how you present it to the animal!

Within the literature review I recognized that 2,500 years ago Heraclitus identified monotony as an unnatural condition for living things, and I hypothesized that removing or minimizing it within the diets of livestock by incorporating 'functional diversity' could reduce existing production, environment, and welfare inefficiencies, which the results of this thesis support. In other words... Heraclitus was right.

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