Impact of dietary inclusion of field peas (*Pisum sativum*) on the production, rumen fermentation, and composition of rumen bacterial community of lactating dairy cows

By

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ABSTRACT

The objective of this study was to determine the effects of partially substituting a corn grain-based concentrate diet with coarsely ground field peas on milk yield and composition, blood metabolites, rumen fermentation parameters, and rumen bacterial community composition in lactating dairy cows. This study used 12 mid-lactation Holstein cows in a repeated 3 × 3 Latin square experimental design with three 21-d periods. The third week of each period is designated for sampling. The cows were fed either a control total mixed ration (TMR) or TMR mixed diets containing field peas at a 3.9% dry matter (DM) inclusion (LP) or a 7.8% DM inclusion (HP). Increasing inclusion rates of field peas in the diet linearly increased ruminal ammonia nitrogen ($P = 0.02$), milk urea nitrogen ($P < 0.01$), plasma urea ($P = 0.01$), and total concentration of ruminal branched-chain volatile fatty acid ($P = 0.05$) concentrations. Dry matter intake (DMI) increased quadratically ($P = 0.03$), and milk fat percentage increased linearly ($P = 0.05$) with higher milk C16:0 fatty acids and lower C18:2 isomers without impacting milk yield, protein, lactose levels, ruminal pH, and short-chain volatile fatty acid (VFA) concentrations. However, increasing inclusion rates of field peas in the diet linearly decreased ($P < 0.01$) the total tract digestibility of DM, crude protein (CP), and neutral detergent fiber (NDF). Although the alpha diversity indices of the rumen bacterial community remained unchanged, a comparison of the Euclidean distances of the rumen bacterial community revealed a trend ($P = 0.07$) between cows fed field pea diets and those in the control group. Additionally, differential abundance analyses revealed changes ($P < 0.05$) in the relative abundances of several bacterial genera and ASVs in response to the field pea diets. This study demonstrated that field peas (up to 7.8% DM) can replace corn grain-based concentrate diets as a potential alternative protein and energy source for lactating dairy cows. Their inclusion can

enhance microbial protein degradation and milk fat percentage without negatively affecting milk production and rumen fermentation.

Keywords: field peas, Holstein lactating dairy cows, milk production, rumen fermentation, rumen bacterial community composition

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LIST OF ABBREVIATIONS

ADF	Acid Detergent Fiber
AIA	Acid Insoluble Ash
ANF	Antinutritional Factor
ANOVA	Analysis of Variance
ADC	Apparent Total Tract Digestibility Coefficient
ASV	Amplicon Sequence Variant
BHBA	Beta Hydroxybutyrate
BCFA	Branched Chain Fatty Acid
CH_4	Methane
CO_2	Carbon Dioxide
CLR	Centered Log Ratio
Cq	Threshold Cycle
CFU	Colony Forming Unit
CP	Crude Protein
DDGS	Dried Distillers Grains with Solubles
LSM	Least Squares Mean
DM	Dry Matter
DMI	Dry Matter Intake
E	Efficiency
H2	Hydrogen
HCL	Hydrochloric Acid
H_2SO_4	Sulphuric Acid
IDF	Insoluble Dietary Fiber
MCP	Microbial Crude Protein
MUFA	Monounsaturated Fatty Acid
MUN	Milk Urea Nitrogen
NDF	Neutral Detergent Fiber
NEFA	Non-Esterified Fatty Acid
NEL	Net Energy for Lactation
NFC	Non-Fiber Carbohydrate
NGS	Next Generation Sequencing
NH_3-N	Ammonia Nitrogen
N_2O	Nitrous Oxide
NPN	Non-Protein Nitrogen
PCA	Principle Component Analysis
PCR	Polymerase Chain Reaction
PDI	Purine Derivative Index
PERMANOVA	Permutational Multivariate Analysis of Variance
PUFA	Polyunsaturated Fatty Acid
qFISH	Quantitative Fluorescence in Situ Hybridization

qPCR	Quantitative Real Time Polymerase Chain Reaction
RDP	Rumen Degradable Protein
RUP	Rumen Undegradable Protein
SCFAs	Short Chain Fatty Acids
SDF	Soluble Dietary Fiber
VFA	Volatile Fatty Acid
TMR	Total Mixed Ration

CHAPTER 1: GENERAL INTRODUCTION

Providing animals with nutritious and high-quality feed is crucial for their health and productivity (Trūpa et al., 2018). The high crude protein (CP) concentration of canola meal and soybean meal makes them primary protein supplements in the diets of lactating dairy cows in Western Canada and the United States (Khorasani et al., 2001; Mulrooney et al., 2009; Pereira et al., 2017), but their cost and availability are affected by fluctuations in global market prices and climate conditions (Jezierny et al., 2010; Trostle, 2008). Therefore, there is a growing global interest in exploring legumes, such as field peas (*Pisum sativum*), as alternative protein sources for ruminants in regions where these legumes are grown. This approach also aim to partially replace genetically modified protein sources, such as soybean meal, within the food chain (Froidmont and Bartiaux-Thill, 2004; Ruzic-Muslic et al., 2014).

After the common bean (*Phaseolus vulgaris*), field peas rank as the world's second most important pulse crop (Tar'an et al., 2004). Field peas are palatable and a rich source of CP and starch, along with essential vitamins and minerals (Khorasani et al., 2001; National Research Council, 2021). Field peas are rich in lysine, but deficient in methionine (N. Singh, 2017). Like other legumes, field peas possess antinutritional factors (ANFs), including α-galactosides, tannins, phytates, and trypsin inhibitors that can limit their nutritional values (Arribas et al., 2019; Vidal-Valverde et al., 2003), However, their effects can be minimized through proper processing methods such as extrusion, which reduce some of the ANFs and enhance the protein and carbohydrate digestibility and nutrients absorption (Bessada et al., 2019; Muzquiz et al., 2012).

Ruminants have a symbiotic relationship with complex microbial communities in the rumen, including various species of bacteria, fungi, protozoa, and archaea, collectively known as the rumen microbiota (Pitta et al., 2016). Microbial fermentation in the rumen is crucial for

fulfilling the protein and energy needs of ruminants by producing microbial crude protein (MCP) and volatile fatty acids (VFA), primarily acetate, propionate, and butyrate (Palmonari et al., 2024), Therefore, the composition and functionality of the rumen microbial community influence ruminants' health and production performance. Microbial fermentation in the rumen generates VFA, which supply approximately 70% of the ruminants' daily energy requirements (Bergman, 1990). Additionally, a large portion of amino acids (AA) absorbed through the small intestine of ruminants are provided by MCP produced in the rumen (60%-85%), while the rest is provided through dietary AA that bypass the rumen degradation (Gruninger et al., 2019). High-producing lactating dairy cows require diets that include sufficient rumen undegradable protein (RUP), providing essential AA for milk production (Zagorakis et al., 2015). However, field peas have a high content of rumen degradable protein (RDP), up to 75% of CP (DM basis), which may limit their inclusion rates in the diets of high-producing lactating dairy cows (Goelema et al., 1998). To reduce the ruminal degradability of dietary protein and enhance the quantity and quality of bypass protein, various processing methods, particularly the heat treatment of protein feeds, are used to improve the availability of AA to the small intestine (Galméus, 2012).

Several studies, including those conducted in Canada, have investigated the effects of partially substituting feedstuffs such as soybean meal, canola meal, corn grain, and barley grain with field peas as a potential alternative source of protein and energy in total mixed ration (TMR) -based lactating dairy cow diets. These studies examined the impact on milk yield and composition, and rumen fermentation parameters (Corbett et al., 1995; Khorasani et al., 2001; Masoero et al., 2006; Pereira et al., 2017; Petit et al., 1997; Vander Pol et al., 2008). Previous research have shown that peas can be incorporated into lactating dairy cow diets up to 25% of DM inclusion rates as a partial replacement for energy and protein supplements without negative effects on production

parameters (Khorasani et al., 2001; Petit et al., 1997; Vander Pol et al., 2008). However, it is recommended to include peas at 15%–20% DM in concentrate mixes or 7%–12% DM in TMR in lactating dairy cow diets (Marx and Schroeder, 2002).

Petit et al. (1997) reported that inclusion of 20.2% extruded or raw peas (DM basis) partially substituting corn grain and soybean meal in a corn grain-based concentrate diet for lactating dairy cows increased DMI, milk urea nitrogen (MUN), and nitrogen excretion in urine, compared to the cows on the control diet, without affecting milk production. However, cows that were fed extruded peas exhibited higher digestibility of DM (DMD) and nitrogen than those on the control or raw pea diets, resulting in cows on extruded peas tending to have higher milk protein percentages compared to the cows fed the control or raw peas (Petit et al., 1997). Another study evaluated the impacts of substituting ground barley and soybean meal with 10% to 30% DM of peas in a barley grain-based concentrate diet for lactating dairy cows (Khorasani et al., 2001). They reported that this substitution had no impact on DMI and milk production. However, as the inclusion rates of field peas increased, the ruminal pH declined, accompanied by an increase in rumen ammonia nitrogen (NH_3-N). Additionally, the rumen proportion of butyrate, valerate, total branched-chain volatile fatty acids (BCVFA; isobutyrate + isovalerate), and total VFA increased (Khorasani et al., 2001). Masoero et al. (2006) substituted soybean meal and barley grain with 10.3% DM of raw, extruded, and expanded peas in a corn grain-based concentrate diet of lactating dairy cows. They reported that extruded peas led to a higher milk yield than control, raw, and expanded peas (Masoero et al., 2006). In another study, partially substituting corn grain and soybean meal with 15% DM of coarsely ground peas in lactating dairy cows did not influence DMI, milk production and composition, and purine derivatives excretion (Vander Pol et al., 2008). However, the inconsistent results observed in previous studies may be due to variations in

inclusion rates of field peas, the specific ingredients replaced by field peas, processing methods, field peas particle size, milk production levels and lactation stages. According to a study by Petit et al. (1997), the particle size of field peas affects their degradability in the rumen. Despite this, there has been few research exploring the impact of field peas particle size, particularly grinding on the degradability of pea protein in the rumen (Bayourthe et al., 2000).

Previous research have elucidated that diet is among the key determinants shaping the composition of the rumen microbiota (Malmuthuge and Guan, 2017; Palmonari et al., 2024). While several studies have investigated the impact of partially replacing peas in the diets of lactating dairy cows on their production performance (Corbett et al., 1995; Khorasani et al., 2001; Petit et al., 1997), the effects of field pea inclusion on the composition and functionality of the rumen microbial population remain poorly understood. The objective of the current study was to investigate the impacts of the partial replacement of a corn grain-based concentrate diet with field peas on DMI, milk production and composition, rumen fermentation parameters, blood metabolites, and the composition of the rumen bacterial community.

CHAPTER 2: LITERATURE REVIEW

2.1 INTRODUCTION

The northern plains of Canada and the United States provide a suitable environment for growing field peas (P. R. Miller et al., 2002). Due to their high CP and starch concentration, lower-grade peas or pea by/co-products that do not meet human consumption standards can be marketed to the livestock feed industry at lower prices (Gilbery et al., 2007). Therefore, interest in using field peas as alternative protein and energy sources in dairy cows feed has increased, leading to several studies evaluating their inclusion in lactating dairy cow diets (Khorasani et al., 2001; Pereira et al., 2017; Vander Pol et al., 2009). The varying results observed in previous studies may be attributed to variations in dietary inclusion rates, the ingredients replaced by field peas, processing methods, particle size of field peas, and factors such as milk production levels and lactation stages. The high ruminal degradability of pea protein can limit its inclusion rate in dairy cow diets due to the risk of excessive rumen NH_3-N production, and it may not meet the RUP requirements of high-yielding dairy cows (Goelema et al., 1998; Petit et al., 1997). Additionally, peas contain ANFs such as tannins, trypsin inhibitors, and phytic acid, which may negatively affect nutrient digestibility and absorption (Bessada et al., 2019). To enhance their nutritive value, various processing methods have been applied. One effective technique for enhancing the nutritional value of grain legumes, such as peas, is extrusion, a thermal processing method. This process reduces ANFs through high temperatures and decreases the rumen degradability of pea protein (Babatunde et al., 2023; Galméus, 2012; Masoero et al., 2005; Petit et al., 1997; Walhain et al., 1992).

The rumen microbiota can ferment a wide range of nutrients, including those that are indigestible to humans (Shabat et al., 2016). Rumen microbes break down rumen-degradable dietary proteins into AA, which are subsequently deaminated into BCVFA and ammonia. Ammonia is then used to synthesize MCP, which provides 60% to 85% of the protein absorbed in the ruminants' small intestine (Keum et al., 2024). In addition, differences in fat concentration and fatty acid profiles of corn, canola meal, soybean meal, and peas may affect how substituting these ingredients with peas in the concentrate diet of lactating dairy cows influences the milk fatty acid profile (Barrera-Arellano et al., 2019; Lewinska et al., 2015; Villalobos Solis et al., 2013). Soybean meal and canola meal, the primary oilseeds in Canada, contain more than 35% and 20% oil, respectively (Villalobos Solis et al., 2013), while field peas have a low fat concentration, ranging from 1.5% to 3.7% (Yoshida et al., 2007) and corn has a slightly higher level, ranging from 3% to 5% depending on the cultivar (Barrera-Arellano et al., 2019). Linoleic acid (C18:2) constitutes a major fatty acid found in soybean oil and pea oil, making up around 54% and 46% of their total fatty acids, respectively. In contrast, oleic acid (C18:1) dominates canola oil, accounting for approximately 61% of its total fatty acids (Hartwig and Kilen, 1991; Lewinska et al., 2015; Villalobos Solis et al., 2013).

Dietary protein and fatty acid concentrations play an important role in dairy cows' productivity and milk composition, particularly milk protein and milk fat percentage, which are key indicators of milk quality (Kudlinskienė et al., 2016; Schingoethe, 2017; Soyeurt et al., 2006). Therefore, this literature review aims to summarize the effects of inclusion of field peas, both raw and processed through different methods such as grinding, cracking, rolling, and extrusion, in lactating dairy cow diets. The primary focus is on the potential of including field peas as alternative sources of protein and energy compared to conventional feeds (soybean meal, canola meal, corn,

and barley grain), particularly their impacts on production performance and rumen fermentation. Additionally, the chapter highlights limited literature (one published research) available on the inclusion of field peas in lactating dairy cow diets on rumen bacterial community composition.

2.2 THE IMPORTANCE OF ALTERNATIVE PROTEIN FEEDSTUFF IN THE DAIRY INDUSTRY

Ruminants are crucial in providing essential nutrition, including milk and meat, for a growing human population (Hunter et al., 2017). Ruminants can convert forages indigestible to humans and monogastric animals into edible products, making them unique compared to monogastrics (Shabat et al., 2016). The high-quality protein content of soybean meal and canola meal, characterized by their high CP concentration and well-balanced AA profile, makes them the primary protein supplements in lactating dairy cow diets across Canada and the United States (Khorasani et al., 2001; Mulrooney et al., 2009; Pereira et al., 2017). However, the importance of the dairy industry for food security, the critical role of protein feeds in dairy diets, the high cost of protein feeds, and the competition for high-quality protein ingredients between human and animal nutrition sectors have driven a growing demand for nontraditional, locally produced protein sources, such as field peas as an alternative source of protein and energy for animal nutrition (Cargo-Froom et al., 2022; Vander Pol et al., 2008).

Cultivating pulse crops, such as peas, not only serves as a high-quality feed resource for dairy farmers but also supports the sustainability of farming systems. Leguminous plants have the ability to fix atmospheric nitrogen and produce nitrogen in symbiosis with root rhizobia, which are nitrogen-fixing bacteria found in the soil and form legume root nodules. This process reduces

the need for nitrogen fertilizers, thereby lowering the fossil energy costs associated with their production and application and consequently, it helps to reduce greenhouse gas emissions, especially nitrous oxide (N_2O; Watson et al., 2017). The cultivation of pulse crops, such as peas is economically similar to cereals and has the added benefit of being more environmentally friendly (Vidal-Valverde et al., 2003), helping to preserve and improve soil fertility (Courty et al., 2015; R. J. Singh et al., 2007). Additionally, non-genetically modified soybean meal is priced twice as high as genetically modified soybean meal (Zagorakis et al., 2015), and there are public concerns about the use of genetically modified feeds in the food chain (Domingo and Giné Bordonaba, 2011). For organically certified dairy cows, soybean meal, which poses a higher risk of genetic modification, can be substituted with peas (Froidmont and Bartiaux-Thill, 2004). Therefore, field peas have become of interest for dairy rations due to their high nutritional value, low production cost, availability, and potential environmental sustainability (Barac et al., 2010).

2.3 FIELD PEAS CHARACTERIZATION

2.3.1 PRODUCTION AND CONSUMPTION OF FIELD PEAS

Field peas, cool-season pulse crops in the *Fabaceae* family (also known as *Leguminosae*), are the most widely cultivated pea species globally (Windsor et al., 2024). The yellow cotyledon species is the most common field pea, followed by the green cotyledon species (Latvia University of Life Sciences and Technologies et al., 2020). The optimum growth temperature for field peas ranges from 12°C to 18°C, although they can tolerate cold temperatures as low as 7°C during germination and growth (Gilbery et al., 2007; P. R. Miller et al., 2002; Reveglia et al., 2025). The growth of field peas is more affected by climatic conditions than by soil nutrient levels (Huang et

al., 2017), therefore, growing field peas can be a potential alternative source of protein, particularly during the cool season in regions unsuitable for soybean meal cultivation (P. R. Miller et al., 2002). In contrast, the optimal soil temperature for soybean meal germination is between 24.2°C and 32.8°C (Tyagi and Tripathi, 1983).

The Northern Plains of Canada and the United States provide a suitable environment in terms of temperature for growing field peas (Gilbery et al., 2007; P. R. Miller et al., 2002). In 2023, Saskatchewan accounted for the highest pea production in Canada at 48.8%, followed by Alberta at 42.8% and Manitoba at 8.4% (Quality of Western Canadian Peas in 2023). Among globally produced grain legumes, field peas rank fourth in production, followed by soybeans, peanuts, and dry beans (Yoshida et al., 2007). While field peas are mainly cultivated for human consumption, the animal feed industry represents a secondary market for lower-grade peas and pea by-products incorporated into animal diets (Gilbery et al., 2007; Kowk, 2022). Livestock feed accounts for the majority of the approximately 35% of Canadian peas consumed domestically (Thiessen, 2004). In addition, European agricultural systems acknowledged that pulse crops by providing protein-rich resource have the potential to reduce European dependence on imported protein (Watson et al., 2017).

2.3.2 NUTRITIONAL COMPOSITION AND FEEDING VALUES OF FIELD PEAS

Field peas are nutritious legumes containing highly degradable proteins, starches, fibers, fats, vitamins, and minerals (Bessada et al., 2019).

2.3.2.1 Protein and Starch Content

Field peas are rich in CP (20-25% DM) and starch (24-49% DM), making them a valuable ingredient in animal feed by providing both protein and energy (National Research Council, 2021; Shanthakumar et al., 2022; Windsor et al., 2024). However, Tzitzikas et al. (2006) noted that field pea CP concentration can vary widely, ranging from as low as 13.7% DM to as high as 38.3% DM. Additionally, field peas are rich in lysin and arginine; however, they have low levels of methionine, cysteine (sulfur-containing AA), and tryptophan (Lallés, 1993; Singh, 2017; Stein et al., 2016). Therefore, when including field peas in a lactating dairy cows diet, it is crucial to balance the AA in the formulation to ensure that all essential AA are provided in the right proportions for optimal production performance (Masoero et al., 2006). Field peas with light green cotyledons exhibited higher lysine content than those with dark green or yellow cotyledons (Vidal-Valverde et al., 2003). Different field pea cultivars vary in chemical composition, particularly in their protein and starch concentrations (Tzitzikas et al., 2006). For instance, the Santana cultivar contains 25% DM of CP and 49% DM of starch, while the Hardy cultivar contains 22% DM of CP and 52% DM of starch (Titze et al., 2021; Zuber et al., 2019). Montan, Danto, AC Tamor, Fluo, Celeste, and Titan are among the field pea cultivars grown in Western Canada (Wang et al., 1998). Additionally, the nutrient composition of a field pea cultivar, including its fatty acid profile and AA content, can vary across different growing locations (Reveglia et al., 2025). Given that their protein consists of 85-100% albumins and globulins (buffer-soluble protein fractions), field peas are highly soluble and readily degradable in the rumen (Wilkins and Jones, 2000).

2.3.2.1.1 Rumen Degradability

Up to 75% of pea protein is categorized as RDP (Goelema et al., 1998), making peas less suitable for grass silage-based diets but better suited for medium- to high-starch diets, where they provide a readily available energy source (Wilkins and Jones, 2000). Combining peas, which have a high RDP concentration, with diets rich in fermentable carbohydrates such as those containing grain-based diets (whole, silage or by-products), can help ensure that rumen microbes have the energy necessary for growth and for the degradation of pea protein (Reynolds and Kristensen, 2008; Wilkins and Jones, 2000). A previous study reported that the Dexter variant (a white-flowered pea) has 67.7% rapid RDP, while the Dolores variant (a colored-flowered pea) has 59.1% (Titze et al., 2021).

2.3.2.2 Fat Content

Field peas have low levels of crude fat, ranging from 1.5% to 3.7% DM (Yoshida et al., 2007). Palmitic acid (C16:0) is the most abundant saturated fatty acid in field peas, which constitutes approximately 6.76% of the total fatty acid content. Additionally, oleic acid (C18:1 *cis*-9) is the predominant monounsaturated fatty acid (MUFA), comprising approximately 31% of total fatty acid content. Linoleic acid (C18:2) is the most abundant polyunsaturated fatty acid (PUFA) in field peas, which represents approximately 46% of the fatty acid profile (Villalobos Solis et al., 2013).

2.3.2.3 Fiber Content

Field peas are an excellent source of protein, starch, dietary fiber, especially insoluble fiber (Wang et al., 2008). Field peas contain 12–24% DM of fiber, including 2% to 9% soluble fiber

and 10% to 15% insoluble fiber (Shanthakumar et al., 2022). Field peas contain cellulose content ranging from 5.2% to 7.7% DM and hemicellulose content from 2.3% to 9.5% DM (Jezierny, 2009).

2.3.2.4 Comparison with Soybean Meal, Canola Meal, Corn Grain, and Barley Grain

Field peas contain less CP concentration (24.3%) compared to soybean meal (48.0%) and canola meal (41.5%). They also have a lower concentration of RUP, at 15% compared to 33% for soybean meal and 32% for canola meal (National Research Council, 2021). According to a previous study, the *in situ* nitrogen solubility of field peas (290 g/kg) was significantly higher than that of soybean meal (135 g/kg; Vander Pol et al., 2009). Both soybean meal and field pea proteins provide high levels of essential AA, comparable to those present in cows' milk. Field pea protein contains 7.2% lysine, comparable to the 6.1% found in soybean meal, and significantly higher than that of corn, which contains 3% (Lallés, 1993; Vander Pol et al., 2008). The methionine content is 0.8% in peas, 1.3% in soybean meal, and 2% in corn grain (National Research Council, 2021).

Field peas have a higher starch content than soybean meals, but they contain lower levels of starch than barley grain (Valentine and Bartsch, 1990). Peas contain approximately 1.2 times the net energy value of soybean meal and 0.9 times that of corn grain (Beyer et al., 2003). Pea starch degrades in the rumen at a rate comparable to corn starch but slower than barley, which has a degradation rate of 82–95%, compared to 56% for pea starch (Cerneau and Michalet-Doreau, 1991; Corbett et al., 1995). Field peas contain 7.9% acid detergent fiber (ADF), while soybean meal has 7.2% and canola meal has 20.2%. Additionally, the neutral detergent fiber (NDF) content in field peas is 12.2%, compared to 11.1% in soybean meal and 29% in canola meal (National Research Council, 2021).

Peas contain crude fat concentration of 2% DM compared to 1.8% DM found in soybean meal (National Research Council, 2021). Linoleic acid (C18:2) is the most abundant fatty acid in pea oil (46%) and soybean oil (54%), whereas oleic acid (C18:1) is the primary fatty acid in canola oil, making up approximately 61% of its total fatty acids. For corn oil, the dominant fatty acids include linoleic acid, which accounts for 34-53% of the total fatty acids, and oleic acid, which comprises 22-53% (Hartwig and Kilen, 1991; Villalobos Solis et al., 2013; Yoshida et al., 2007). In regions where field peas are cultivated, they are used as substitutes for protein sources, such as soybean and canola meals, and energy sources, such as corn and barley grains in lactating dairy cow diets without adversely influencing milk production or composition (Khorasani et al., 2001; Petit et al., 1997; Vander Pol et al., 2008).

2.3.2.5 Antinutritional Factors

Field peas are highly nutritious (high in protein, starch, fiber, vitamins and minerals), but their nutritional benefits can be diminished by ANFs. These include protein-based ANFs, such as protease inhibitors, as well as non-protein ANFs, such as phenolic compounds (tannins and phytic acid; Bessada et al., 2019). These ANFs interfere with nutrient absorption and digestion through various mechanisms. Protease inhibitors, such as trypsin inhibitors, disrupt proteolytic enzymes and reduce protein digestibility (Campos-Vega et al., 2010). Field peas with yellow cotyledons exhibited higher trypsin inhibitor activity than those with green cotyledons (Vidal-Valverde et al., 2003). Phytic acid binds to minerals, proteins, and enzymes, reducing their solubility and functionality. Condensed tannin levels in white-flowered pea cultivars are 100 times lower than those in colored-flowered cultivars; however, white-flowered cultivars still contain trypsin inhibitors (Bastianelli et al., 1998). Overall field peas contain ANFs, but their negative effects can

be minimized through proper processing methods such as extrusion (Bessada et al., 2019). Nevertheless, some ANFs found in pulse grains, such as field peas, are bioactive compounds with health benefits, antioxidant activity, which may outweigh their negative effects (Arribas et al., 2019; Campos-Vega et al., 2010). A previous study reported a positive correlation between the total phenolic content in legume seeds, such as field peas, and their antioxidant activity (Amarowicz et al., 2004).

2.3.2.6 Processing Method

Previous studies have employed various processing methods for pulse crops, including grinding, pelleting, extrusion, boiling, and radiofrequency to enhance their protein and starch digestibility as well as their nutritional values (Babatunde et al., 2023; Cargo-Froom et al., 2022). The extrusion process is a high-temperature and short-time treatment, whereas pelleting is conducted at lower temperatures but can still alter various components of the feed, such as protein denaturation and the destruction of some vitamins (Svihus and Zimonja, 2011). Dehulling can reduce trypsin inhibitor activity in field peas, although cooking is more effective at decreasing trypsin inhibitor activity than dehulling (Wang et al., 2008). Research has shown that grinding and pelleting can influence the CP concentration of two varieties of Canadian peas. In a study by Cargo-Froom et al. (2022), grinding increased the CP concentration of Amarillo and Dun peas without altering the AA composition, while no differences were observed between finely and coarsely ground peas. Conversely, pelleting improved the CP and specific AA concentrations including phenylalanine, leucine, and lysine, but led to a decrease in histidine levels than whole and ground peas. Additionally, the study noted that grinding reduced the crude fiber content of Dun peas, likely due to the reduction of hulls throughout the grinding process (Cargo-Froom et al.,

2022). Additionally, in ruminants, most AA absorbed in the small intestine are supplied by microbial protein produced in the rumen (Kung and Rode, 1996). A previous study reported that a 60:40 ratio of RDP to RUP in the diet is optimal for highly productive dairy cows (Zagorakis et al., 2015). To reduce the ruminal degradability of dietary protein and subsequently to increase the outflow of dietary AA to the small intestine, different processing methods, especially heat treatment, are employed. Extrusion can decrease the ruminal degradability of pea protein and reduce the ANFs of legumes more cost-effectively than other heating methods, such as baking or autoclaving (Alonso et al., 1998; Galméus, 2012; Masoero et al., 2005). Petit et al. (1997) found that lactating dairy cows offered diets that included extruded peas showed higher DM and nitrogen digestibility, leading to an increased milk percentage compared to those fed raw peas or no pea. Another study reported that feeding extruded peas increased milk yield compared to the control group, as well as the groups fed raw or expanded peas, in lactating dairy cows (Masoero et al., 2006). Furthermore, a previous study demonstrated that extrusion decreased the starch content and increased the soluble dietary fiber content of two varieties of Canadian peas (Babatunde et al., 2023).

2.4 IMPORTANCE OF RUMEN MICROBIAL ECOSYSTEM IN RUMINANTS

2.4.1 SYMBIOTIC RELATIONSHIP BETWEEN THE RUMEN MICROBIAL COMMUNITY AND RUMINANTS

Ruminants have the ability to efficiently utilize a wide variety of nutrients, including those that are indigestible to humans and monogastric animals (Shabat et al., 2016). The digestive system of ruminants maintains a symbiotic relationship with a complex microbial community in the rumen,

reticulum, omasum, and large intestine. The microbial community of the rumen includes diverse species of bacteria, fungi, protozoa, archaea, and viruses collectively known as the rumen microbiota (Pitta et al., 2016). In terms of proportions detected in metagenomics studies, bacteria constitute approximately 95% of the rumen microbial community, archaea account for 0.3% to 3.3%, and eukaryotes, including fungi and protozoa, make up approximately 1.5% (Castillo-Lopez et al., 2014; Mizrahi, 2013). However, a recent study utilizing metatranscriptomics revealed an increased involvement of eukaryotes in rumen microbial degradation (Comtet-Marre et al., 2017). The findings indicate that in addition to the fibrolytic bacteria in the rumen, such as *Prevotella*, *Ruminococcus*, and *Fibrobacter*, fungi and ciliate protozoa also contribute to the degradation of polysaccharides. The rumen supplies nutrients and provides an anaerobic environment that supports the growth of rumen microbes. In return, these microbial symbionts perform crucial functions for ruminants, such as the degradation and fermentation of complex polysaccharides and other major dietary nutrients, the production of VFA and MCP as primary sources of energy and protein, and the synthesis of vitamins and various metabolites that support the physiology and health of cattle (Palmonari et al., 2024).

2.4.1.1 Bacteria

Bacteria are the most functionally crucial microorganisms and dominate the rumen microbial population, comprising 10^{10}–10^{11} colony-forming units (CFUs) per ml of rumen fluid. The most abundant phyla are Bacteroidota and Bacillota (Castillo-Lopez et al., 2018; Derakhshani et al., 2017) and the most abundant families are Prevotellaceae, Lachnospiraceae, Ruminococcaceae, and Fibrobacteriaceae (De Menezes et al., 2011). The rumen microbiota plays a key role in breaking down a wide variety of feed components, producing VFA, ammonia, which

supports microbial activity and MCP, along with other by-products such as carbon dioxide (CO_2), lactate, and methane (CH_4; Krause et al., 2003). Studies used real-time qPCR (quantitative polymerase chain reaction) for quantification of several bacterial species in the rumen (Klieve et al., 2003; Stevenson and Weimer, 2007). Stevenson and Weimer (2009) quantified different rumen bacterial taxa using the qPCR method in lactating cows. They reported that the genus *Prevotella*, belonging to the phylum Bacteroidota, dominated the rumen bacterial community, comprising 42–60% of the total 16S rRNA gene copies in their rumen samples. However, known ruminal *Prevotella* species, including *Prevotella bryantii*, *Prevotella brevis*, and *Prevotella ruminicola*, represented only 2–4% of the overall bacterial population. The proportions of *Selenomonas ruminantium*, *Ruminococcus flavefaciens*, *Fibrobacter succinogenes*, and *Succinivibrio dextrinosolvens* ranged from 0.5% to 1% of the total bacterial rRNA gene copies in the rumen. The abundance of rRNA gene copies for *Ruminococcus albus*, *Streptococcus bovis*, *Butyrivibrio fibrisolvens*, and *Megasphera elsdenii* was less than 0.03%. (Stevenson and Weimer, 2007). Research using amplicon sequencing of the 16S rRNA gene identified differences in bacterial community composition between rumen solid and liquid fractions in both TMR- and pasture-fed dairy cows (De Menezes et al., 2011). Petri et al. (2012), using real-time qPCR, reported that the abundance of *Ruminococcus* spp., *Fibrobacter succinogenes*, and *Selenomonas ruminantium* was higher in the solid phase than in the liquid phase of the rumen in cattle fed a high-concentrate diet.

Bacteria are the initial group to adhere to feed particles and play a crucial role in the digestion of various dietary components, particularly fibrous components (Huws et al., 2013). In addition, novel genes from the Actinomycetota, Fibrobacterota, and Pseudomonadota phyla were identified in the rumen (Stewart et al., 2019). Up to 75% of rumen bacteria have been characterized

as being attached to feed particles, with the remainder free-floating in the rumen fluid (Koike et al., 2003; Tedeschi and Nagaraja, 2025). Rumen bacteria are classified into different groups based on their functionality, including fibrolytic, amylolytic, proteolytic, saccharolytic, and lactate-utilizing species, each specializing in the digestion and degradation of specific nutrients such as fiber, starch, protein, sugars, and fat. Among these groups, starch and sugar degraders from the amylolytic and saccharolytic categories constitute a large proportion of the ruminal bacterial community (Deusch et al., 2017). In contrast, the proportion of fibrolytic bacteria is smaller, despite their crucial role in fiber degradation within the digestive system (Puniya et al., 2015).

2.4.1.2 Archaea

Methanogens, classified under the phylum Euryarcheota, the most abundant archaeal community in the rumen (Mizrahi, 2013). The most abundant methanogens in the rumen are *Methanobrevibacter gottschalkii* and *Methanobrevibacter ruminantium* (Gruninger et al., 2019). Methanogens are classified into hydrogenotrophic, methylotrophic, or acetoclastic archaea. The majority are hydrogenotrophic, utilizing CO_2 and hydrogen (H_2) produced during microbial fermentation of nutrients in the rumen to synthesize CH_4. Methylotrophic methanogens can grow using hydrogen along with methyl groups derived from methylamines or methanol. Acetoclastic methanogens, such as *Methanosarcina* spp. and *Methanosaeta* spp., produce CH_4 from acetate but are extremely rare (Cholewińska et al., 2020; Henderson et al., 2015). According to the study by Henderson et al. (2015), 77.7% of archaea in the rumen were hydrogenotrophic methanogens, 22.1% were methylotrophic, and acetoclastic methanogens accounted for less than 0.015%. Methanogens can exist freely in the rumen fluid, attached to particles of feed, live as symbionts with rumen protozoa from genera *Entodinium, Epidinium*, *Ophryoscolex,* and *Polyplastron*, cross-

feed with anaerobic rumen fungi, or are attached to the epithelium of rumen (Bauchop and Mountfort, 1981; Janssen and Kirs, 2008; Sharp et al., 1998).

2.4.1.3 Protozoa

In the rumen, the majority of protozoa are ciliates from the phylum Ciliophora (Newbold et al., 2015). Rumen protozoa may digest fiber, although this is not well understood (Devillard et al., 2003). While not critical to the functioning of the rumen, the absence of these microorganisms can affect digestion of feed and CH_4 emissions due to their symbiotic relationship with methanogens (Mizrahi and Jami, 2018). Entodiniomorphid protozoa are protozoal species that can engulf starch granules, inhibiting their rapid fermentation by lactic acid-producing bacteria and therefore contributing to the maintenance of buffering capacity within the rumen (Bonhomme, 1990). Additionally, they can degrade dietary proteins and prey on bacteria, which helps prevent the rapid proliferation of bacteria (Nagaraja, 2016).

2.4.1.4 Fungi

Anaerobic rumen fungi are important for fiber degradation in the rumen by producing fiber-degrading enzymes, Cellulases, hemicellulases, and glycosidases, and are particularly abundant in high-fiber diets (Cholewińska et al., 2020). In addition, they can physically penetrate and break down plant material, thereby increasing the available surface area for microbial colonization (Hess et al., 2020). Research has revealed that Neocallimastigomycota is the most abundant fungal phylum in the rumen of cattle, with *Piromyces, Anaeromyces, Cyllamyces, Neocallimastix,* and *Orpionmyces* as the dominant genera within this phylum (Wang et al., 2019). A study

conducted by Bernalier et al. (1993) showed that *Ruminococcus flavefaciens* can inhibit the cellulolytic activity of *Neocallimastix frontalis,* which is the best-studied fungi in the rumen. Therefore, there may be interactions between rumen bacteria and anaerobic fungi (Bernalier et al., 1993).

2.4.2 ROLE OF RUMINAL BACTERIA IN PROTEIN AND NITROGEN METABOLISM IN THE RUMEN

Rumen microbes allow ruminants to digest rumen-degradable dietary proteins, breaking them down into peptides and AA. The AA are then deaminated to produce ammonia as the main end-product and BCVFA as the primary by-product of the deamination of branched-chain amino acids, along with other products such as CO_2 and H_2. Ammonia, a non-protein nitrogen source, is then used by rumen microbes to synthesize MCP. This process is driven by energy produced through carbohydrate fermentation and efficient recycling of urea and nitrogen between the blood and the rumen (Reynolds and Kristensen, 2008). MCP is degraded in the abomasum and reaches the small intestine in the form of AA and peptides, serving as a crucial AA source for ruminants, ranging from 60% to 85% of the total AA absorbed in their small intestine (Keum et al., 2024). In ruminants, amino acids absorbed in the small intestine are supplied either by microbial protein produced in the rumen or through dietary protein that bypasses rumen degradation (Gruninger et al., 2019). Clark et al. (1992) summarized the AA composition of 441 bacterial samples isolated from the rumen of animals fed different treatments and demonstrated that ruminal bacteria are a valuable source of essential AA, despite significant differences in their composition. However, they are still limited in some AA, including methionine and histidine. Rumen microbial community

plays an essential role in contributing up to 70% of the animal's energy supply and accounting for 60-85% of MCP absorbed in the small intestine, making the rumen microbiome a compelling research topic in recent years (Brulc et al., 2009; Petri et al., 2012). However, when the amount of RDP in the diet exceeds the needs of rumen microbes, excessive ammonia is produced which is then absorbed into the bloodstream, converted to urea in the liver, and ultimately excreted in the urine and feces, contributing to environmental pollution (Hristov et al., 2011; Savari et al., 2018).

In ruminants, the recycling of urea-nitrogen between the rumen and liver plays a crucial role in improving nitrogen utilization efficiency (Lapierre and Lobley, 2001). Urea-nitrogen recycling serves as an important nitrogen source for microbial protein synthesis in the rumen, particularly when dietary rumen degradable protein or nitrogen is limited (Chalupa, 1973). Previous studies have indicated that reducing dietary CP can negatively impact milk yield and milk protein yield, which diminishes the profitability of dairy producers (Cabrita et al., 2011; Chibisa and Mutsvangwa, 2013). To support high milk yield, 16% DM is the dietary CP concentration recommendation for lactating dairy cows (National Research Council, 2021). In addition, as RDP concentration increases and exceeds the needs of rumen microbes, rumen NH_3-N concentration also increases (Reynolds and Kristensen, 2008). However, elevated ruminal NH_3-N concentration decrease the epithelial permeability in the rumen to urea-nitrogen transfer, thereby negatively impacting urea-nitrogen recycling from the blood to the rumen in cattle (Kennedy and Milligan, 1980). Furthermore, ureolytic ruminal epithelial bacterial activity, which facilitates urea-nitrogen transfer into the rumen, is also negatively correlated with ruminal NH_3-N concentration (Cheng and Wallace, 1979). Therefore, the nitrogen degradability of feedstuffs plays a key role in determining the amount of energy wasted through the conversion of blood ammonia into urea and its excretion in urine and feces (Miller and Baig, 2002).

Rumen microbes primarily utilize ammonia as their main source of nitrogen for growth and microbial protein synthesis, though some species, such as some strains of *Bacteroidota ruminicola* (Pittman and Bryant, 1964), can also use preformed AA and peptides if available in the rumen (Bryant and Robinson, 1962). Chikunya et al. (1996) reported that microbial protein synthesis, assessed through urinary purine derivatives, increased in sheep-fed beet pulp supplemented with casein (as a source of AA) compared to those supplemented with urea, as a source of non-protein nitrogen (NPN). This suggests that preformed AA and peptides in the rumen can stimulate microbial protein synthesis more effectively than urea. However, they found no effect of AA supplementation on microbial protein synthesis in sheep-fed grass hay. These findings indicate that AA supplementation enhances microbial protein synthesis only when diets contain a substantial proportion of rapidly fermentable carbohydrates, which serve as a source of energy for this process (Chikunya et al., 1996; Soto et al., 1994). Pereira et al. (2017) also reported a higher DMI and milk protein concentration for lactating dairy cows fed a diet containing 25% field peas, which partially replaced ground corn and completely replaced urea (1.3%), compared to those fed a diet with urea but without the inclusion of field peas. Since the CP concentration of field peas is highly rumen-degradable (Vander Pol et al., 2009), when pea protein undergoes proteolysis, it produces ammonia, similar to the ammonia generated from urea degradation, along with preformed AA and peptides. This may enhance microbial protein synthesis in lactating dairy cows fed field peas compared to those fed only a NPN source, such as urea, when the diet contains sufficient rapidly fermentable carbohydrates (Pereira et al., 2017). Members of the phylum Bacillota, such as *Clostridium sticklandii*, *Selenomonas ruminantium*, and *Eubacterium ruminantium*, along with *Lactobacillus fermentum*, as well as members of the phylum Pseudomonadota, such as *Ruminobacter amylophilus* can degrade dietary protein by producing

proteolytic enzymes (Cholewińska et al., 2020; Palmonari et al., 2024). The genus *Prevotella*, within the Bacteroidota phylum is also capable of degrading a wide range of nutrients, including proteins, starch, sugars, pectin, and cellulose (Osorio-Doblado et al., 2023; Parra et al., 2022; Petri et al., 2012). They are also capable of synthesizing MCP using NPN sources such as ammonia and urea (Palmonari et al., 2024).

2.4.3 ROLE OF RUMINAL BACTERIA IN CARBOHYDRATES AND FIBER METABOLISM IN THE RUMEN

Dietary fibrous plant material is rapidly colonized in the rumen by fibrolytic bacteria and the plant cell wall begins to degrade within 15 min of entering the rumen (Edwards et al., 2007). The rumen microbial community is capable of producing cellulolytic and hemicellulolytic enzymes to break down complex fibrous feed components, such as cellulose and hemicellulose, into fermentable carbohydrates, VFA, and other by-products such as CO_2, lactate, and CH_4 (Gruninger et al., 2019; Jami et al., 2014). Anaerobic rumen fungi function as extensions of bacterial biofilms attached to fiber particles, enhancing fiber digestion through both physical breakdown and enzymatic degradation (Hess et al., 2020). The VFA generated through microbial fermentation of dietary carbohydrates in the rumen pass through the rumen epithelium and serve as the main source of energy for ruminants, supplying approximately 70% of the daily metabolizable energy required for growth, development, and milk production (Bergman, 1990; Keum et al., 2024). Therefore, ruminal carbohydrate degradation is an essential aspect of feed evaluation in ruminant nutrition (Plaizier et al., 2008).

Previous studies have identified Gram-negative *Fibrobacter succinogenes* and Gram-positive species such as *Butyrivibrio fibrisolvens*, *Ruminococcus albus*, and *Ruminococcus flavefaciens* as primary rumen bacteria with cellulolytic function, known for their ability to produce cellulases (Jami et al., 2014; Jenkins et al., 2008; Krause et al., 2003). Previous research also reported that *Prevotella bryantii* and *Prevotella ruminicola* work synergistically with cellulolytic bacteria to break down plant cell walls (Cholewińska et al., 2020). Although *Prevotella* is not a major cellulolytic bacteria, it is capable of producing a variety of xylanases for the degradation of xylan (Krause et al., 2003). The fermentation of plant-based carbohydrates by rumen microorganisms is complex, as compounds produced by one microorganism during fermentation often serve as substrates for others (Dehority, 1991). Dietary starch can be utilized by common amylolytic and fibrolytic rumen bacteria, including *Ruminobacter amylophilus*, *Streptococcus bovis*, several *Prevotella* species, *Selenomonas ruminantium*, *Butyrivibrio fibrisolvens*, and *Succinomonas amylolytica*. Furthermore, Golder et al. (2014) found that the abundance of Streptococcaceae, such as *Streptococcus bovis*, and Veillonellaceae, such as *Megasphaera elsdenii* and *Veillonella parvula* increased with the incorporation of readily fermentable carbohydrates in cattle diets.

2.4.4 ROLE OF RUMINAL BACTERIA IN FAT BIOHYDROGENATION AND MILK FAT SYNTHESIS

Although the ruminant diet is high in PUFA, unsaturated fatty acids can be converted into saturated fatty acids by rumen microbes as a result of biohydrogenation (Jenkins et al., 2008), affecting the fatty acid composition of milk (Carreño et al., 2019). Bacteria are the primary ruminal

microorganisms responsible for fatty acid biohydrogenation (Carreño et al., 2019). Some unsaturated fatty acids, including conjugated linoleic acids (CLAs), primarily C18:2 *cis-9 trans-11*, as well as C18:1 *cis-9* (oleic acid), C18:1 *trans-11* (vaccenic acid), and C18:3n-3 (α-linolenic acid), are beneficial to human health, such as cancer prevention and reduction of atherosclerosis (Chilliard et al., 2007; Palmquist et al., 2005); however, increasing their concentration in milk remains challenging due to biohydrogenation in the rumen (Jenkins et al., 2008). *Butyrivibrio fibrisolvens* plays a central role in the biohydrogenation of fatty acids (Lourenço et al., 2010) and other bacteria recognized as active in biohydrogenation include members of *Selenomonas* (Vasta et al., 2009), *Butyrivibrio* (Schofield et al., 2001), *Pseudobutyrivibrio* (Polan et al., 1964), *Megasphaera elsdenii* (Y. J. Kim et al., 2002), and *Propionibacterium* (Fujimoto et al., 1993) genera. Increasing dietary PUFA in ruminants may increase PUFA proportion in milk by enhancing the bypass of PUFA from the rumen or by altering microbial metabolic activity (Lourenço et al., 2010).

2.4.5 ROLE OF RUMEN MICROBIOME ON PRODUCTION EFFICIENCY

Feed efficiency refers to how efficiently the feed consumed by animals is converted into food products such as milk or meat (Mizrahi, 2012). Adjusting the diet can positively affect the rumen microbiota composition, leading to improvements in efficiency of feed and cattle production (Wang and Guan, 2022). Jami et al. (2014) demonstrated a correlation between milk yield and composition and the abundance of the rumen bacterial community. Specifically, a negative correlation was identified between the abundance of *Prevotella* genus in the rumen and milk fat yield, while a positive correlation was identified between the abundance of

Coriobacteriales order and milk lactose content (Jami et al., 2014). Li and Guan. (2017) reported higher rumen microbial activity at both the compositional and functional levels between two beef cattle groups with low and high feed efficiencies, based on residual feed intake using metatranscriptomics. They reported that the relative abundance of Lachnospiraceae, Lactobacillaceae, and Veillonellaceae was higher in the efficient group compared to the inefficient one. The study also found that four AA metabolism pathways related to lysine, cysteine, methionine, histidine, and tyrosine were more active in the feed-efficient group of beef cattle compared to the inefficient group, suggesting a higher level nitrogen metabolism activity in the rumen of efficient cattle (Li and Guan, 2017), a pattern also observed in dairy cows (Shabat et al., 2016).

2.4.6 DYNAMICS OF RUMEN MICROBIAL COMMUNITY IN RESPONSE TO DIETARY CHANGES

While the core functionality of the rumen microbiome is redundant and stable, the composition of the rumen microbiome could be dynamic and rapidly adapt to dietary changes (Weimer, 2015). The microbial composition of the rumen alters in response to abrupt shifts in the diet composition (Chen et al., 2021; Maia et al., 2010). For instance, a higher abundance of *Prevotella* was observed in the rumen of beef cattle fed a diet with low protein (8.8% CP) compared to those on a diet with high protein (13.5% CP), likely due to their ability to obtain nitrogen from various nutrient sources such as ammonia and urea, which enhances nitrogen recycling (Parra et al., 2022). Another study, using qPCR, reported that *Streptococcus bovis* was

one of the dominant bacteria in the rumen of dairy cows fed grain-rich diets (Khafipour et al., 2009).

2.5 FIELD PEA INCLUSION IN DAIRY COW DIETS

2.5.1 EFFECTS ON DMI, MILK PRODUCTION, MILK COMPOSITION, AND MILK FATTY ACID PROFILES

Corbett et al. (1995) reported that partially substituting canola meal and barley grain and completely replacing soybean meal and wheat ground with 12.5% field peas (DM basis) in an isoenergetic and isonitrogenous barley grain-based concentrate diet, balanced for rumen-undegradable protein, increased milk fat percentage but decreased overall milk yield of high-producing lactating dairy cows fed pea diet (Corbett et al., 1995). In another study, high-producing dairy cows in early lactation were fed isonitrogenous and isoenergetic corn grain-based concentrate diets supplemented with either soybean meal, raw or extruded peas (Petit et al., 1997). The inclusion of 20.2% peas (DM basis) partially replacing corn grain and soybean meal significantly increased DMI and MUN concentrations without affecting milk yield or composition. However, diets containing extruded peas increased milk protein percentage (Petit et al., 1997). Khorasani et al. (2001) incrementally replaced ground barley and soybean meal with 10–30% DM peas in lactating dairy cow diets containing an equal ratio of forage to concentrate (DM basis) had no effect on DMI and milk yield. Masoero et al. (2006) replaced soybean meal and barley grain with 10.3% raw, extruded, or expanded peas in a corn grain-based concentrate diet of lactating dairy cows. The study found that extruded peas improved milk yield compared to the control, raw, and expanded pea diets. Vander Pol et al. (2008) partially replaced soybean meal and corn grain

with 15% DM of coarsely ground field peas in the diet of high-producing lactating dairy cows with an average yield of 35 kg milk daily and found that diet did not affect DMI, milk yield, and composition. However, when 15% DM of rolled peas were added to a diet to replace soybean meal and corn grain, the DMI and milk yield were decreased (Vander Pol et al., 2009). Pereira et al. (2017), replaced 1.3% urea and 36% corn grain with 25% DM of field peas in a corn grain-based concentrate diet of lactating dairy cows. Compared to cows fed urea, cows fed field peas showed increased DMI, milk production, milk protein concentration, and urinary purine derivatives. In the rumen, the degradation of CP from field peas produces not only ammonia but also preformed AA and peptides that enhance microbial protein synthesis and increase milk protein concentration. However, the inclusion of field peas did not affect the milk fat percentage. The conflicting outcomes of the above studies may be attributed to variations in diet formulation, inclusion rates of peas, processing techniques used to incorporate peas into dairy cow diets, particle size of field peas, and factors related to milk production levels and lactation stages.

2.5.2 EFFECTS ON RUMEN FERMENTATION CHARACTERISTICS AND BLOOD METABOLITES

Vander Pol et al. (2009) revealed that the inclusion of 15% DM of rolled or coarsely ground field peas in lactating dairy cow diets by replacing soybean meal and corn grain, does not affect the total or individual short-chain VFA concentrations, such as acetate, propionate, and butyrate, in ruminal fluid. However, Khorasani et al. (2001) observed a linear increase in butyrate concentration when ground barley and soybean meal were incrementally replaced with 10-30% DM of ground peas. Additionally, a linear decline in rumen pH and an elevation in rumen NH_3-N

concentration were observed in pea-fed cows, likely attributed to higher dietary protein concentration and the deamination of AA. Similarly, higher butyrate concentrations were reported in the rumen of dairy cows receiving field peas intrarumenally compared to those given barley grain or lupins (Valentine and Bartsch, 1987).

2.5.3 EFFECTS ON RUMEN BACTERIAL DIVERSITY AND COMPOSITION

Several studies have been conducted to assess the effect of field peas on milk production and composition and rumen fermentation parameters in lactating dairy cows, but other than one study, there appears to be no research to date that investigates the effects of the inclusion of field peas on the composition of rumen bacterial community in lactating dairy cows. Castillo-Lopez et al. (2018) partially replaced a barley-based concentrate with 11.4% raw or extruded flaxseed-based products containing 37.8% field peas in the diets of lactating Holstein cows to evaluate the rumen bacterial population using 16S rRNA gene amplicon sequencing. They observed that inclusion of flaxseed-based products did not significantly affect the relative abundances of the dominant rumen bacterial phyla (Bacteroidota, Bacillota, Pseudomonadota, and Mycoplasmatota), families (Prevotellaceae, unclassified Bacteroidales, Veillonellaceae, and Lachnospiraceae), and genera (*Prevotella*, *unclassified Bacteroidales*, *Succiniclasticum*, and *unclassified Clostridiales*). Due to the importance of rumen microbiota in the fermentation of feedstuff and the production performance of lactating dairy cows, there is a need to better explore its dynamics in response to varying dietary inclusion rates of field peas.

2.5.4 COMMON MOLECULAR TECHNIQUES USED TO STUDY THE DYNAMICS OF RUMEN MICROBIAL COMMUNITY

Culture-based techniques have traditionally been used to isolate and study the rumen bacterial community (Hungate et al., 1964). Robert Hungate was a pioneer in the study of rumen microbiology (De Menezes et al., 2011). However, culture-dependant methods are only capable of detecting approximately 10-20% of the rumen bacterial population, making them inadequate for comprehensively characterizing the complex rumen bacterial community (Kong et al., 2010; Zeineldin et al., 2018). Advances in molecular techniques, such as metataxonomics, metagenomics, metatranscriptomics, metaproteomics, and metabolomics, have significantly enhanced our understanding of the rumen microbiome. Recent advancements in sequencing technology, such as improved read lengths, reduced costs, and increased throughput, allow researchers to conduct comprehensive studies, sequencing multiple gene regions of thousands of samples rather than being limited to a small number of amplicons (D'Amore et al., 2016). While 16S rRNA gene amplicon sequencing is commonly used to analyze rumen bacterial composition, shotgun metagenomics, metatranscriptomics, metaproteomics, and metabolomics, can covey comprehensive information about both the rumen bacterial community composition and the functional potential and metabolic pathways of their genes (De Menezes et al., 2011; Li et al., 2016). Moreover, some studies have employed culture-independent approaches such as quantitative fluorescence in situ hybridization (qFISH). However, qFISH has limitations compared to next-generation sequencing (NGS), as the probes may not target all rumen 16S rRNA genes (Duenas et al., 2004). The 16S rRNA gene, found in nearly all bacteria, is the most widely used genetic marker for assessing microbial communities, capable of generating large numbers of sequences within a few hours (Sanjorjo et al., 2023). Additionally, qPCR is a useful method for

quantifying specific genes or microbial taxa; however, it is not suitable for comprehensively investigating microbial communities as a whole (Khafipour et al., 2016). The design of primer pairs in this method requires ensuring they are specific to the target gene and exclusive, to prevent amplification of unintended genes (Tajima et al., 2001). Since understanding the complex rumen microbial community using a single approach is insufficient, several studies combine molecular techniques such as metagenomics, metatranscriptomics, metaproteomics, and metabolomics for a deeper analysis of the rumen microbiome (Comtet-Marre et al., 2017; Deusch et al., 2017).

2.6 CONCLUSIONS

The importance of the dairy industry for global food security, along with the considerable expense of protein feeds underscores the growing demand for locally produced protein sources such as field peas. Field peas offer the potential to be an alternative protein and energy source in lactating dairy cow diets, replacing more traditional feed sources such as soybean meal, canola meal, corn, or barley grains. However, the high RDP concentration of field peas may limit their inclusion rate in the diets of high-producing lactating dairy cows as they may not provide sufficient RUP to meet the cows' requirements and could result in excessive production of rumen NH_3-N, blood urea, and MUN, therefore reducing the efficiency of nitrogen utilization and negatively impacting milk yield and composition. Research indicates that field peas can be included up to 25% DM in lactating dairy cow diets. However, it has been recommended that peas be included in lactating dairy cows' feed formulations at a DM rate of 7-12% in TMR without negatively affecting production performance, though their impact on the structure of rumen microbial ecosystem and its relationship with the rumen fermentation profile remains poorly understood.

Effects of dietary field pea inclusion on rumen pH, VFA, NH_3-N, and urinary purine derivative excretion of lactating dairy cows suggest potential impacts on the rumen microbiota composition and functionality. Consequently, further investigation is needed to explore the effects of varying inclusion rates of field peas on rumen fermentation profile and microbiota composition.

2.7 HYPOTHESES AND OBJECTIVES

2.7.1 HYPOTHESES

1) Partial replacement of a corn grain-based concentrate diet with field peas in the diet of lactating dairy cows would increase the degradation and deamination of dietary protein in the rumen, resulting in increasing concentrations of NH_3-N, BCVFA, MUN, and blood urea without negatively affecting rumen fermentation (total VFA and pH) and milk production performance (milk yield and composition).

2) This replacement would also change the rumen bacterial community composition in favor of increasing the proportion of proteolytic bacteria.

2.7.2 OBJECTIVES

The overall objectives of this study were to investigate the influence of a partial replacement of a corn grain-based consecrate diet with field peas on DMI, the yield and composition of milk, rumen fermentation, blood metabolites, and the rumen bacterial community composition in lactating dairy cows.

CHAPTER 3: Impact of inclusion of field peas (*Pisum sativum*) on the production, rumen fermentation, and composition of rumen bacterial community of lactating dairy cows

3.1 ABSTRACT

Field peas are a potential source of protein and energy for livestock; however, their high rumen degradable protein concentration may limit their inclusion rates, and the impact on rumen bacterial community composition remains poorly understood. The objective of this study was to investigate the effects of partial substitution of a corn grain-based concentrate diet with field peas (*Pisum sativum*) on milk production, composition, and fatty acid profile, rumen fermentation, blood metabolites, and the rumen bacterial community composition of lactating dairy cows. Twelve Holstein dairy cows in mid-lactation were assigned to a repeated 3 × 3 Latin square experimental arrangement of treatments, comprising 21-d periods, with sampling and data collection performed during the final 7 days. Cows were fed either a control total mixed ration (TMR), or a TMR with 3.9% DM (LP) or 7.8% DM (HP) of field peas. The control diet consisted of 17.4% crude protein (CP), 28.0% starch, and an estimated 1.67 Mcal/kg of net energy for lactation (DM basis). The inclusion of field peas in the diet increased dry matter intake (DMI) and milk fat percentage, from 23.7 to 24.8 kg/d and from 4.16 to 4.38%, respectively, without affecting the milk yield, milk protein, and milk lactose. Increasing the inclusion rate of field peas in the diet increased the ruminal ammonia nitrogen (from 6.17 to 8.67 mg/dl), milk urea nitrogen (from 11.4 to 12.7 mg/dl), and plasma urea nitrogen (from 4.20 to 4.46 mmol/L). The total tract digestibility of DM, CP, and neutral detergent fiber (NDF) decreased as the inclusion rate of field peas in the diet increased. There were no significant changes in ruminal pH, as well as in total and individual short-chain volatile fatty acid (VFA) concentrations while the total concentration of ruminal branched-chain VFA (isobutyrate and isovalerate) tended to increase linearly from 1.26 to 1.42 mmol/L with

increasing inclusion rates of field peas in the diet. The proportion of C16:0 milk fatty acid increased while reducing the proportions of the C18:2 isomers. Inclusion rates of field peas did not influence alpha diversity indices of rumen bacterial community. However, a comparison of the beta-diversity of the microbiota revealed a divergence ($P = 0.07$) between cows fed field pea diets and those in the control group. Differential abundance analyses also revealed changes ($P < 0.05$) in the relative abundances of several bacterial ASVs belonging to Prevotellaceae, Lachnospiraceae, and Ruminococcaceae in response to the field pea diets. Additionally, the relative abundance of Selenomonas decreased while that of Shuttleworthia increased in response to the inclusion of field peas in the diet. This study demonstrated that field peas (up to 7.8% DM) can be a potential alternative protein and energy source in corn grain-based concentrate diets of lactating dairy cows. Their inclusion in the diet can maintain feed efficiency, milk production, nitrogen utilization, and rumen function while supporting DMI and milk fat synthesis. Given their nutritional value and local availability, field peas could reduce reliance on traditional protein and energy sources like soybean meal and corn grain, offering a more sustainable feeding strategy for dairy farmers.

3.2 INTRODUCTION

The Northern Plains of Canada and the United States provide a suitable environment for growing field peas (Gilbery et al., 2007; P. R. Miller et al., 2002). In 2023, the production of field peas in Western Canada was 2598 tonnes (Quality of Western Canadian Peas, 2023). Field peas are available in various forms, including raw, split, ground, cracked, toasted, extruded, pea hulls, pea cream and pea screenings (Kowk, 2022; Offner et al., 2003). Due to their high crude protein (CP) and starch concentrations, the livestock feed industry represents a potential market for lower-

grade peas that do not meet human consumption requirements (Gilbery et al., 2007). Therefore, interest in using field peas as cattle feed has increased, leading to several studies on their inclusion in dairy cow diets (Pereira et al., 2017; Vander Pol et al., 2009).

Corbett et al. (1995) partially substituted canola meal and barley grain and completely replaced soybean meal and wheat ground with 12.5% field peas (DM basis) in a barley grain-based concentrate diet and found an increase in milk fat percentage but a decline in milk production of lactating dairy cows on a pea diet. Petit et al. (1997) provided isonitrogenous and isoenergetic, corn grain-based concentrate diets supplemented with either soybean meal, raw peas, or extruded peas to lactating dairy cows in the early stages of lactation. The study found that the inclusion of 20.2% peas (DM basis) as a partial replacement for corn grain and soybean meal increased dry matter intake (DMI), milk urea nitrogen (MUN), and nitrogen excretion in urine compared to cows fed the control diet. However, cows fed extruded peas had higher digestibility of DM (DMD) and nitrogen compared to those on the control or raw peas, resulting in a tendency for higher milk protein percentage than in cows on the control or raw peas (Petit et al., 1997). Khorasani et al. (2001) replaced ground barley and soybean meal with 10-30% DM field peas in a lactating dairy cow diet with a 50:50 forage-to-concentrate ratio (DM basis), finding no impact on DMI or milk yield. However, as the inclusion rates of peas in the diet increased, there was a decrease in ruminal pH and an increase in rumen ammonia nitrogen (NH_3-N), as well as in ruminal butyrate, valerate, total short-chain volatile fatty acid (VFA), and branched-chain volatile fatty acid (BCVFA) concentrations (Khorasani et al., 2001). In another study, partially replacing soybean meal and corn grain with 15% DM of coarsely ground field peas in a diet of high-yielding lactating dairy cows did not affect DMI, milk production, composition, and purine derivatives excretion (Vander Pol et al., 2008). However, when either 15% DM of rolled peas or coarsely ground field peas were

added to lactating cow diets to replace corn grain and soybean meal, these inclusions of peas increased rumen NH_3-N and reduced DMI and milk yield, suggesting that rumen nitrogen metabolism may be affected. The reduced DMD of the rolled pea diet in this study was explained by an increase in undigested pea particles in the feces (Vander Pol et al., 2009). Pereira et al. (2017) conducted a study in which 1.3% urea was completely replaced, and 36% corn grain was partially substituted with 25% DM field peas in a corn grain-based concentrate for lactating dairy cows. The results revealed that cows fed field peas had increased DMI, higher milk yield, improved milk protein content, and higher urinary excretion of purine derivatives compared to those fed urea and lower inclusion rate of corn grain (Pereira et al., 2017). The variation in the effects of including field peas in lactating dairy cow diets observed across studies can be attributed to several factors, including differences in inclusion rates, processing methods, stage of lactation, milk yield, and the response of the rumen microbial community to dietary inclusion of field peas. Since previous studies have indicated that field peas can affect the proportions of ruminal VFA and NH_3-N, two of the main products of microbial metabolism of nutrients in the rumen, better understanding of the underlying dynamics of the rumen bacterial community's response to different inclusion rates of field peas is essential. Studies have demonstrated that field peas can be included in dairy cow diets as a replacement for starch and protein-based concentrates up to 25% DM (Corbett et al., 1995; Khorasani et al., 2001; Masoero et al., 2006; Pereira et al., 2017; Petit et al., 1997; Vander Pol et al., 2009). However, inclusion rates of 15-20% DM of field peas in concentrate mixes and 7-12% DM in total mixed rations (TMR) have been recommended (Marx and Schroeder, 2002). Higher inclusion rates of field peas may lead to an excessive intake of rumen-degradable protein (RDP) and starch while creating deficiencies in rumen-undegradable protein (RUP) and essential

amino acids (AA). This imbalance can pose challenges for milk protein synthesis in high-yielding dairy cows (Corbett et al., 1995; Khorasani et al., 2001; Pereira et al., 2017).

In this study, we hypothesized that partially replacing a corn grain-based concentrate diet with field peas as an alternative protein source with a high RDP concentration would increase protein degradation and deamination in the rumen of lactating dairy cows. This, in turn, would increase NH_3-N and total concentration of BCVFA in the rumen without negatively impacting milk production. We further hypothesized that the inclusion of field peas would change the composition of rumen bacterial community in favor of increasing the proportions of proteolytic bacteria. The objectives were to investigate the impact of two inclusion rates of filed peas (3.9% and 7.8% DM) on the production performance, milk composition, blood metabolites, rumen fermentation, and rumen bacterial community composition of lactating dairy cows.

3.3 MATERIALS AND METHODS

3.3.1 ANIMALS, DIETARY TREATMENTS, AND EXPERIMENTAL DESIGN

The experimental procedures were approved by the Fort Garry Campus Animal Care Committee of the University of Manitoba (with approval number F22-010), and animal care followed the guidelines of the Canadian Council on Animal Care (2017).

Cows were accommodated in individual tie stalls lined with rubber mats and chopped straw bedding, with ad libitum access to water. Cows were provided with exercise for 1 h, three times per week, following the morning milking and prior to the morning feed delivery. Five primiparous and seven multiparous lactating Holstein cows from the Glenlea Research Station of the University

of Manitoba (mean ± SD, 161 ± 57.0 DIM; 672 ± 27.0 kg BW) were used. Cows were blocked based on their parity, total milk production in the previous lactation, and days in milk in a repeated 3 × 3 Latin square experimental design of treatments with three experimental periods. Each experimental period lasted 3 weeks with sampling and data collection occurring in the third week of each period. Cows were assigned to one of the following treatments: (1) a total mixed ration (TMR) without peas (Control treatment); (2) a TMR including 3.9 % DM of peas (LP treatment); and (3) a TMR containing 7.8% DM of peas (HP treatment). Whole field peas were obtained from Zeghers Seed Inc., Holland, MB, Canada. Before feeding, the peas were ground in an IFA Roller Grinder System (Iowa Farm Automation, Stanley, IA). The particle size distribution of the ground peas was determined in duplicate using the Pen Sate Particle Separator with 19, 8, and 1.18 mm screens and a bottom pan (Kononoff et al., 2003). The proportions retained at each level were 7.9%, 48.9%, 20.2%, and 23.0%, respectively. The ingredient composition of the experimental diets, as well as the chemical composition of the experimental diets, dietary forages and field peas are provided in Table 1, Table 2 and Table 3, respectively. The fatty acid composition of the experimental diets and the peas are provided in Supplementary Table S1 in Appendix A. Experimental diets contained similar NE_L, non-fiber carbohydrates (NFC), and macro mineral contents, but the inclusion of filed pea resulted in moderate increases in the dietary concentrations of neutral detergent fiber (NDF) and crude fat, and a moderate decrease in the dietary concentration of CP.

Table 1. Ingredient composition of experimental diets

Item	**Control**	**LP[1]**	**HP[2]**
Ingredient (% DM)			
Corn silage	32.6	36.5	39.7
Straw	3.4	3.6	3.4
Alfalfa silage	29.4	25.1	22.2
APF fat[3]	1.3	1.4	1.4
Supplement[4]	33.6	29.8	25.6
Peas	0	3.9	7.8

[1] LP = 3.9 % DM peas

[2] HP = 7.8 % DM peas

[3]APF Fat Plus, Auburn Laboratories Inc. Penn Valley, CA, USA

[4] Supplement: Corn (ground; 42.5%); Barley (ground; 12.7%); Corn Distillers (11.2%); GoldPass bypass expeller processed soy protein. Natural Proteins Inc. Blumenort, MB, Canada (9.4%); Porcine Meat Meal (7.0%); Canola Meal (4.7%); Sodium Bicarbonate (2.0%); Fat Choice White Mix Rothsay, Winnipeg, MB, Canada (1.9%); Salt - Potash (1.6%); Ground Limestone (1.6%); Feather Meal (1.6%); Soybean Meal (1.6%); Dicalcium Phosphate (0.6%); Magnesium Oxide (0.6%); MB Dairy Ultra Micro Trouw Nutrition Guelph, ON, Canada (36g; 0.5%); RM104, Rumen modifier, Trouw Nutrition Guelph, ON, Canada (0.3%); MHA Methionine Analogue - Novus Saint Charles MO, USA (0.14%); Rumensin, Elanco Animal Health, Greenfield, IN (0.03%); Biopower SC viable yeast product Lallemand Montreal QC Canada (0.03%)

Table 2. Chemical composition of experimental diets

Item	**Control**	**LP[1]**	**HP[2]**
DM, %	56.9	54.8	53.2
CP, % DM	17.4	17.2	16.9
NDF, % DM	35.5	37.3	37.3
ADF, % DM	19.4	19.1	19.1
AIA, % DM	1.14	1.23	1.36
Crude fat, % DM	4.5	4.6	4.8
Ash, % DM	8.1	8.2	8.1
NFC, % DM	41.8	41.4	41.1
Starch, % DM	28.0	28.6	29.3
Ca, % DM	0.95	1.00	0.96
P, % DM	0.40	0.40	0.44
Mg, % DM	0.37	0.37	0.37
Na, % DM	0.39	0.39	0.39
K, % DM	1.48	1.48	1.51
NE_L, Mcal/kg	1.67	1.67	1.67

[1] LP = 3.9 % DM peas
[2] HP = 7.8 % DM peas

Table 3. Chemical composition of dietary forages and field peas

Item	Corn Silage	Barley Straw	Alfalfa Silage	Field Peas
DM, %	34.3	93.4	34.4	89.1
CP, % DM	7.3	4.3	19.3	22.5
NDF, % DM	38.0	80.0	32.0	8.21
ADF, % DM	22.5	61.0	27.6	6.56
Crude fat, % DM	2.5	1.8	2.3	1.67
Ca, % DM	0.18	0.22	1.72	0.10
P, % DM	0.22	0.04	0.41	0.42
Mg, % DM	0.14	0.13	0.96	0.14
Na, % DM	0.01	0.14	0.05	0.01
K, % DM	1.06	1.75	2.72	1.04
NE_L, Mcal/kg DM	1.63	0,92	1.53	1.81

3.3.2 DRY MATTER INTAKE AND FEED CHEMICAL COMPOSITION ANALYSES

Cows were fed twice daily at 0930 h and 1600 h, allowing for 5-10% feed refusals. TMR samples were collected once daily throughout each sampling week (from Day 15 to Day 21), and stored at -20 °C, then pooled according to the treatment and period. Individual orts were sampled daily before feed delivery during the sampling weeks (from Day 15 to Day 21), frozen at -20 °C, and then pooled according to the treatment and period. Pooled diet and orts samples were dried in a forced-air oven at 60 °C for 48 h and ground in a Wiley mill with a 1-mm screen (Thomas-Wiley, Philadelphia, PA) for further analyses. The DMI was determined using the weight and DM contents of offered and orts. Samples were analyzed for DM and CP using AOAC method 990.03, as outlined by Horwitz and AOAC International (2006). NDF was analyzed following the method

described by Van Soest et al. (1991), using α-amylase (Sigma No. A3306; Sigma Chemical Co., St. Louis, MO, USA) and sodium sulfite, with an Ankom 200 Fiber Analyzer (Ankom Technology, Fairport, NY, USA). Acid detergent fiber (ADF) was analyzed according to AOAC method 973.18 (AOAC 1990) using the same Ankom 200 Fiber Analyzer. Starch content was measured using a UV method (method 996.11; AOAC 2005). Acid insoluble ash (AIA) was analyzed using AOAC method 920.08 (AOAC 2005). Ether extract and ash analyses were performed using AOAC methods 920.39 (AOAC 1990) and 923.03 (AOAC 2005), respectively. The analysis of K, Ca, Mg, P and Na was carried out using inductively coupled plasma emission spectroscopy (AOAC method 968.08; AOAC 1990) and a Plasma Spectrometer (Thermo Jarrell Ash Corp., Grand Junction, CO, USA) following acid digestion. Net energy for lactation (NE_L) was measured following National Research Council, (2021) recommendations for dairy cows. Rumination time was recorded using rumination sensors (Transponder infrared H-HR-QWES) attached with collars to the cows in the last 7 d of each experimental period.

3.3.3 FECAL SAMPLE COLLECTION AND ANALYSES

Fecal grab samples were collected two times daily at 0900 h and 1500 h for 5 d in the final week of each experimental period (from Day 16 to Day 20), according to the collection procedure by Li et al. (2012). Around 250 g of fecal grab samples were collected from the rectum of each cow and stored at -20 °C, then pooled according to the treatment and period. The samples were thawed and subsequently dried in an oven at 60 °C for 7 d before being ground using a CT 293 Cyclotec Mill with a 1-mm screen (Cyclotec, 293 Sample mill, Foss Tecator). The fecal samples were then placed in whirl-pak bags and kept at room temperature for further analysis. Fecal

samples were tested for DM and CP concentration using a modification of AOAC 990.03 method, while NDF, ADF, and AIA were determined according to AOAC 942.05 (Horwitz and AOAC International, 2006).

3.3.4 APPARENT TOTAL TRACT DIGESTIBILITY COEFFICIENTS OF NUTRIENTS

The DM, CP, NDF, and AIA contents of experimental diets and pooled feces of individual cows in the final week of each experimental period were utilized for the measurement of the apparent total tract digestibility coefficients (ADC) of nutrients. The AIA content in the diets was applied as an internal marker for assessing digestion according to the procedure by Mc Geough et al. (2010) and Van Keulen and Young (1977), and ADC of DM, CP, and NDF were measured based on the following equations:

$$\text{DMD} = 1 - \left(\frac{\text{AIA \% DM in diet}}{\text{AIA \% DM in feces}}\right)$$

$$\text{ADC CP} = 1 - \left[\left(\frac{\text{CP \% DM in feces}}{\text{CP \% DM in diet}}\right) \text{x} \left(\frac{\text{AIA \% DM in diet}}{\text{AIA \% DM in feces}}\right)\right]$$

$$\text{ADC NDF} = 1 - \left[\left(\frac{\text{NDF \% DM in feces}}{\text{NDF \% DM in diet}}\right) \text{x} \left(\frac{\text{AIA \% DM in diet}}{\text{AIA \% DM in feces}}\right)\right]$$

3.3.5 MILK YIELDS AND MILK ANALYSES

Cows were milked twice daily at approximately 0600 h and 1700 h, from Day 15 PM to Day 21 AM during sampling weeks. Milk yields were measured with Tru-Test regulation meters (Westfalia Surge, Mississauga, ON, Canada) and for milk composition analysis, 40 mL pooled

milk samples were collected from the regulation meter, transferred into 50 mL vials, and stored at 4 °C until all four consecutive milkings had been completed. Samples were later warmed at room temperature, vortexed, and aliquots collected in 50 mL vials based upon a yield-to-weight ratio and stored at 4 °C until further milk components analysis at Horizon Laboratories (Winnipeg, MB, Canada). Mid-infrared analysis using a Milk-o-Scan 303AB (Foss Electric, Hillerød, Denmark) was used to analyze the fat, CP, lactose, and MUN concentration of these samples. The same pooling process was followed for milk fatty acid composition analysis, but the milk samples in this case were stored at -20 °C until analysis.

The composition of fatty acids in milk was measured using gas chromatography based on methods reported by Boivin et al. (2013). Milk fat fractions were extracted and methylated as described by Chouinard et al. (1997). Subsequently, analysis was performed with a gas chromatograph (7890A GC; Agilent Technologies Canada Inc., Mississauga, ON) using a 100-m CP-Sil 88 capillary column (0.25-mm i.d., 0.20-µm film thickness; Agilent Technologies Canada Inc.) and a flame ionization detector. The injector temperature was set to 100 °C for 1 min, then increased by 5 °C per min to 220 °C, where it was held for 20 min. Energy Corrected Milk (ECM) in kg per d and feed efficiency were measured using the following equation (Hall, 2023):

$$\text{ECM (kg/d)} = (0.327 \times \text{milk yield (kg/d)}) + (12.95 \times \text{fat yield (kg/d)}) + (7.2 \times \text{protein yield (kg/d)})$$

$$\text{Feed efficiency} = \text{ECM (kg/d)} / \text{DMI (kg/d)}$$

3.3.6 RUMEN FLUID, URINE, AND BLOOD SAMPLE COLLECTION AND ANALYSES

Approximately 10 mL of rumen fluid was collected from each animal once per period at 1500 h on Day 17 (six cows) and Day 18 (six cows) via rumenocentesis according to Duffield et al. (2004). Before the rumen fluid collection, 20 cc of Lidocaine was injected subcutaneously and into the muscle as a local block on each side of the site of entry before cows were administered 10 units of xylazine intravenously. Immediately following collection, pH was measured using an Accumet Basic 15 pH meter (Fisher Scientific, Fairlawn, NJ), fitted with a Sensorex 450C Flat Surface Combination pH/Reference Electrode (Sensorex, Stanton, CA). To prevent cross-contamination, the pH electrode was rinsed with distilled water and wiped clean between samples. Three subsamples (1 mL each) of rumen fluid were then pipetted into three, 2 mL tubes and flash-frozen in liquid nitrogen immediately and stored at -80 °C until subsequent DNA extraction. In addition, 2 mL of rumen fluid was transferred into tubes containing either 0.4 mL of 25% metaphosphoric acid or 0.4 mL of sulphuric acid (H_2SO_4) and kept at -20 °C for analysis of rumen VFA and NH_3-N, respectively.

For the analysis of VFA, frozen rumen samples were thawed and centrifuged at 1,900 × g for 20 min at 4 °C. One mL of the supernatant was transferred into clean gas chromatography vials using a filtered syringe. The vials were subsequently loaded into an autosampler (8410 Varian, Walnut Creek, CA) for VFA analysis using a model 3900 gas chromatograph (Varian, Walnut Creek, CA), following the method outlined by Bhandari et al. (2007). The gas chromatograph injector and detector were programmed to temperatures of 170 °C and 195 °C, respectively, while the column temperatures were programmed to start at 120 °C and reach a maximum of 165 °C. Samples were processed for 4 min, with a subsequent 2-min thermal stabilization phase. Indole-

Phenol Blue Method was used to assess the NH_3-N concentration in rumen fluid samples, as outlined by Novamsky et al. (1974).

Approximately 80 mL of urine was collected twice daily on two non-consecutive days (Day 15 and Day 19) during each sampling period from the middle phase of urination through perirenal stimulation to prompt urination. Following collection, 30 mL of urine was mixed with 2 mL of 3N hydrochloric acid (HCl) to maintain urine pH below 2.0 and reduce nitrogen volatilization. The samples were stored at -20 °C until uric acid, creatinine, and allantoin analysis following the method outlined by Makkar and Chen (2004). To measure microbial protein synthesis indirectly, the Purine Derivative Index was calculated based on creatinine, allantoin, and uric acid concentrations and metabolic body weight (Makkar and Chen, 2004). The index was calculated using the following formula:

Purine Derivative Index = [Uric acid (mmol/L) + Allantoin (mmol/L)]/Creatinine (mmol/L) × kg metabolic weight (BW 0.75)

Blood samples were collected twice daily on two non-consecutive days (Day 15 and Day 19) during each sampling period from the tail vein in 10 mL vacutainer tubes, stored at room temperature for 30 min to allow clotting, and then centrifuged at 1,900 × g for 10 min at 4 °C. The resulting serum was immediately stored at -20 °C for further analyses. Serum sample analysis was performed by Veterinary Diagnostic Services (Manitoba Agriculture, Winnipeg, MB). Concentrations of beta-hydroxybutyrate (BHBA) and non-esterified fatty acids (NEFA) were measured with the RX Monza analyzer with Randox kits (Randox Laboratories Ltd, Crumlin, UK). Additionally, urea and glucose concentrations in serum were measured with the Cobas C 502 analyzer (Roche Diagnostics GmbH, Mannheim, Germany).

3.3.7 DNA ISOLATION AND LIBRARY PREPARATION FOR RUMEN BACTREIAL COMMUNITY ANALYSES

Rumen fluid samples were thawed at 4 °C overnight and extraction of DNA performed using the Quick-DNA™ Fecal/Soil Microbe Miniprep Kit (Zymo Research, Irvine, CA) following the manufacturer's guidelines. The DNA concentration in each sample was assessed using a Qubit dsDNA HS kit (ThermoFisher Scientific, Mississauga, ON, Canada). The DNA samples were stored at -80 °C until further analysis. Purified DNA was used for amplification of the V4 variable region (~250nt in length) of the 16S rRNA gene by polymerase chain reaction (PCR) technique. A total of 2.5 µl DNA was used as template with 12.5 µl of GoTaq® G2 Hot Start Colorless Master Mix, 8.0 µl of nuclease-free water, and 1.0 µl of 5 mM concentration of 515F (GTGYCAGCMGCCGCGGTAA) and 806R (GGACTACHVGGGTWTCTAAT) Illumina adapted primers as outlined by Parada et al. (2016). Three negative controls were included in the PCR reactions by substituting genomic DNA with 2.5 µl of nuclease-free water. The PCR amplifications were performed using the VeritiPro™ Thermal Cycler (Thermo Fisher Scientific Inc., MA, USA) with an initial denaturation at 98 °C for 2 min, then 35 cycles were performed at 98 °C for 15 s, 57 °C for 20 s, and 72 °C for 20 s, with a final extension at 72 °C for 5 min. The resulting products, including negative controls, were visualized on a 1.5% agarose gel to verify PCR amplification. Indexed amplicons from each sample were pooled in equal quantities and sequenced on the Illumina MiSeq platform (Illumina, San Diego, CA, USA).

3.3.8 QUANTITATIVE REAL-TIME PCR

Real-time qPCR was carried out on a Bio-Rad CFX 1000 system with Bio-Rad software (Bio-Rad, Hercules, CA, USA) for absolute quantification of a) bacterial 16S rRNA gene copy numbers, b) *Prevotella genus*, c) *Prevotella bryantii*, d) *Ruminococcus genus*, and e) *Selenomonas ruminantium* using specific primers listed in Table 4. Amplification efficiencies (E) of each primer set and quantification of gene copy numbers were calculated using synthetic gene blocks specific to each primer pair, selected and designed according to the corresponding 16S rRNA gene of each bacterial genus/species deposited in the SILVA database (version 138.1). Gene blocks were synthesized using Integrated DNA Technologies (IDT, USA) and used for the construction of standard curves with concentrations ranging from 10^2 to 10^8 copies. A standard curve was generated for each rumen bacterial lineage by plotting the threshold cycle (Cq) against the logarithm of the DNA quantities. The equation $E = 10^{-1/\text{slope}}$ was applied to determine amplification efficiency. Standard curves were included in every experiment to determine the quantity of each rumen bacterial gene by comparing the sample Cq values to the curve. Each amplification reaction was conducted in a final volume of 20 µl, including 10 µl SsoAdvanced Universal SYBR Green Supermix (Applied Biosystems, Foster City, CA), 1 µl of bovine serum albumin (BSA, 10 mg/ml), 1 µl of each forward and reverse primer (10 µM), 2 µl of DNA template, and 5 µl of nuclease-free water. The thermal cycling program began with an initial denaturation at 98 °C for 2 min, followed by 39 amplification cycles of denaturation at 98 °C for 10 s and annealing/elongation at 60 °C for 15 s. The melt cycle was conducted with a temperature increase from 65 °C to 95 °C, with a 5-s hold at each 0.5 °C increment.

Table 4. Specific primers for quantifying rumen bacteria in real-time qPCR assay of cows fed experimental diets (No peas, Control; 3.9 % DM peas, LP; and 7.8 % DM peas, HP)

Target bacterium	Primer sequence (5′–3′)	T_m[1] (°C)	Amplicon length (bp)	References
Bacterial 16S	F: AAACTCAAAKGAATTGACGG R: CTCACRRCACGAGCTGAC	60	174	Surette lab
Prevotella genus	F: GGTTCTGAGAGGAAGGTCCCC R: TCCTGCACGCTACTTGGCTG	60	121	Stevenson and Weimer (2007)
Prevotella bryantii	F: AGCGCAGGCCGTTTGG R: GCTTCCTGTGCACTCAAGTCTGAC	60	91	Stevenson and Weimer (2007)
Ruminococcus genus	F: GAGTGAAGTAGAGGTAAGCGGAATTC R: GCCGTACTCCCCAGGTGG	60	243	Petri et al. (2013)
Selenomonas ruminantium	F: CAATAAGCATTCCGCCTGGG R: TTCACTCAATGTCAAGCCCTGG	60	138	Stevenson and Weimer (2007)

[1]Annealing Temperature

3.3.9 STATISTICAL ANALYSES

Production parameters, rumen fermentation, blood metabolites, digestibility, milk composition, and urine data were analyzed using a 3 x 3 Latin square design in R (version 4.3.3). The linear model applied was as follows:

$$Y_{kijl} = \mu + B_k + C_{i(k)} + P_j + T_l + e_{kijl}$$

Where Y_{kijl} = observation of dependent variables, μ = overall mean, B_k = fixed effect of block, $C_{i(k)}$ = fixed effect of cow nested within block, P_j = fixed effect of period, T_l = fixed effect of treatments and e_{kijl} = residuals.

To check the validity of the homogeneity of variance and normality assumptions, the Bartlett's test and Shapiro-Wilk test were performed, respectively. A log10 transformation was applied to the response variables that did not meet the homogeneity of variance and normality assumptions. Least Squares Means (LSM) of data were used to assess treatment effects using Linear and quadratic contrasts. Statistical significance was considered at $P < 0.05$.

3.3.10 BIOINFORMATICS ANALYSES

Raw Illumina sequencing reads were analyzed with the DADA2 pipeline (version 1.28.0; Callahan et al., 2016), which was performed in R (Version 4.3.3). Chimeras were filtered to obtain effective tags which were subsequently denoised into amplicon sequence variants (ASV). Taxonomic classifications were assigned to the representative sequences of ASVs using the SILVA database (version 138.1; Quast et al., 2012), (http://www.arb-silva.de/). Prior to downstream bioinformatics analyses, all ASVs assigned to phyla Cyanobacteriota and Chloroplast,

as well as those assigned to genus *Hornefia* were removed as potential sequencing artifacts and/or contaminants.

3.3.10.1 Alpha and beta diversity analyses

Microbiota richness and diversity were determined using the Chao1 index of species richness, and Shannon and inverse Simpson indices of diversity using the "Phyloseq" package in R (version 1.46.0). To evaluate the effects of treatments on the overall composition of the rumen bacterial community, the ASV table was transformed using the centered log-ratio (CLR) approach and used for calculating β-diversity among samples using the Aitchison's distance, measuring the dissimilarity of the composition of microbial communities across samples (Martino et al., 2019). The results were visualized using principal component analysis (PCA). The adonis function from the vegan package in R (version 2.4-6) was used to perform nonparametric permutational multivariate analysis of variance (PERMANOVA) to examine significant differences in β-diversity metrics of rumen bacterial communities between treatment groups. Label permutations (n = 9999) were used in PERMANOVA to assess the distribution of test statistics under the null hypothesis that within-group Aitchison's distance does not significantly differ from between-group distance ($P_{PERMANOVA} < 0.05$).

3.3.10.2 Correlation of production and rumen fermentation parameters with bacterial genera

Spearman's rank correlation analysis of production and fermentation parameters with bacterial genera was performed in R and correlation coefficients were visualized using the corrplot package (version 0.92). Parameters included DMI, milk yield, milk composition, MUN, blood urea,

rumen NH_3-N, blood BHBA, NEFA, short-chain and branched-chain VFA, and the most abundant bacterial genera identified in this study. Pairwise correlations with correlation coefficient > 0.5 and *P*-value < 0.05 were considered significant.

3.3.10.3 Differential abundance analysis of bacterial taxa among treatment groups

Differential abundance analysis of bacterial taxa at the ASV, family, and genus taxonomic ranks was conducted among treatment groups using the MaAsLin2 package (Microbiome Multivariable Associations with Linear Models, version 1.14.1; Mallick et al., 2021). The analysis included CLR transformation of the input ASV table followed by fitting the block, cow, period, and treatment as fixed effect factors in a linear regression model to assess significant differences in the relative abundance of bacterial taxa among treatments at different taxonomic ranks. Differential abundance analysis at the ASV level was limited to those with a minimum relative abundance of 0.005% of the community and detected in at least 10% of samples. At the genus level, differential abundance analysis was limited to those genera with a minimum relative abundance of 0.05% of the community and detected in at least 25% of samples.

3.4 RESULTS

3.4.1 DRY MATTER INTAKE AND BLOOD SERUM METABOLITES

The DMI changed quadratically (*P* = 0.03), with an initial increase from 23.7 kg/d for control to 24.8 kg/d for LP, followed by a decrease to 24.3 kg/d for HP, but the daily duration of rumination was not impacted by treatment (Table 5). Treatment did not affect the blood serum

concentrations of glucose, NEFA, and BHBA, but increasing the inclusion rate of peas linearly increased (P = 0.01) the blood serum concentration of urea from 4.20 to 4.46 mmol/L.

Table 5. Effects of increasing rate of pea inclusion in lactating dairy cow diets on dry matter intake (DMI), rumination time, and blood serum metabolites

	Treatment				**Significance of Contrasts**	
Item	**Control**	**LP[1]**	**HP[2]**	**SEM[3]**	**Linear** ***P-values***	**Quadratic** ***P-values***
DMI, kg/day	23.7	24.8	24.3	0.58	0.11	0.03
Rumination time, min/d	558	561	547	9.48	0.50	0.51
Urea, mmol/L	4.20	4.28	4.46	0.08	0.01	0.57
Glucose, mmol/L	3.64	3.65	3.67	0.03	0.55	0.88
BHBA[4], mmol/L	0.80	0.85	0.85	0.02	0.18	0.41
NEFA[5,6], mmol/L	0.10	0.10	0.08	0.007	0.18	0.21

[1] LP = 3.9 % DM peas

[2] HP = 7.8 % DM peas

[3] SEM = standard error mean

[4] BHBA = beta-hydroxybutyrate

[5] NEFA = non-esterified fatty acids

[6] Data were $\log_{10}$-transformed prior to statistical analysis, but LSM are presented in their original form

3.4.2 APPARENT TOTAL-TRACT DIGESTIBILITY OF NUTRIENTS

Increasing the inclusion of field peas linearly reduced the ADC of DM, CP, and NDF, from 71.4% to 64.7% for DM, 69.7% to 61.8% for CP, and 58.2% to 47.8% for NDF (Table 6).

Table 6. Effects of increasing rate of pea inclusion in lactating dairy cow diets on apparent total tract digestibility coefficients (ADC) of dry matter (DM), crude protein (CP) and neutral detergent fiber (NDF)

	Treatment				Significance of contrasts	
ADC	**Control**	**LP[1]**	**HP[2]**	**SEM[3]**	**Linear *P-values***	**Quadratic *P-values***
DM, %	71.4	69.4	64.7	0.007	<0.01	0.18
CP, %	69.7	67.7	61.8	0.008	<0.01	0.12
NDF, %	58.2	56.1	47.8	0.01	<0.01	0.04

[1] LP = 3.9 % DM peas

[2] HP = 7.8 % DM peas

[3] SEM = standard error of mean

3.4.3 MILK YIELD AND MILK COMPOSITION

The inclusion of filed peas did not influence milk yield, the percentages and yields of milk protein, and milk lactose (Table 7). However, milk fat percentage tended to increase linearly from 4.16-4.38% ($P = 0.05$) in response to the increasing inclusion rate of field peas. The inclusion of field peas in the diet led to a linear ($P < 0.01$) increase in MUN concentration from 11.4 to 12.7 mg/dl. Additionally, there were no differences in ECM and feed efficiency among treatments.

Inclusion of field peas in the diet linearly increased the milk fatty acid proportion of C16:0 by 7.7% (Table 8). In contrast, a linear decline ($P < 0.05$) was observed in the concentrations of the milk fatty acids C18:1 *trans-5*, C18:1 *trans-6-8*, C18:1 *trans-9*, C18:1 *trans-11*, C18:1 *trans-12*, C18:2 *Cis-12, trans-8*, C18:2 *Cis-13, trans-8*, C18:2 *Cis-9, trans-12*, C18:2 *Cis-15, trans-11*, C18:2 *Cis-9, trans-11*, C18:3n3, and total PUFA with increasing inclusion rate of field peas in the

diet. However, as the inclusion rate of peas in the diet increased, there was a linear increase in milk fatty acid proportion of C22:3n3 from 0.006 to 0.008 (Supplementary Tables S2 and S3 in Appendix A).

Table 7. Effects of increasing rate of pea inclusion in lactating dairy cow diets on milk yield, composition, and feed efficiency

	Treatment				Significance of contrasts	
Item	**Control**	**LP[1]**	**HP[2]**	**SEM[3]**	**Linear** ***P-values***	**Quadratic** ***P-values***
Milk yield[4], kg/d	35.8	36.6	35.8	1.18	0.83	0.39
Fat, %	4.16	4.22	4.38	0.10	0.05	0.58
Fat, kg/d	1.49	1.53	1.56	0.06	0.13	0.83
Protein, %	3.40	3.40	3.38	0.04	0.26	0.84
Protein, kg/d	1.21	1.24	1.20	0.04	0.72	0.30
Lactose, %	4.62	4.60	4.63	0.02	0.41	0.21
MUN[5], mg/dl	11.4	11.2	12.7	0.39	<0.01	0.05
ECM[6], kg/d	39.8	40.7	40.5	1.35	0.48	0.52
Feed efficiency[7]	1.68	1.64	1.65	0.02	0.54	0.42

[1] LP = 3.9 % DM peas

[2] HP = 7.8 % DM peas

[3] SEM = standard error of mean

[4] Data were $\log_{10}$-transformed prior to statistical analysis, but LSM are presented in their original form

[5] MUN = milk urea nitrogen

[6] ECM = energy corrected milk

[7] Feed efficiency = ECM (kg/d) / DMI (kg/d)

Table 8. Effects of increasing rate of pea inclusion in lactating dairy cow diets on proportions of *de novo* and C16 milk fatty acids (g/100 g of FA)

	Treatment				Significance of contrasts	
Item	**Control**	**LP[1]**	**HP[2]**	**SEM[3]**	**Linear** ***P-values***	**Quadratic** ***P-values***
C6:0	1.92	1.92	1.95	0.05	0.80	0.85
C8:0	1.12	1.10	1.12	0.03	0.99	0.65
C10:0	2.45	2.40	2.47	0.07	0.86	0.54
C10:1	0.26	0.26	0.25	0.008	0.67	0.99
C11:0	0.06	0.07	0.07	0.005	0.53	0.28
C12:0	2.98	2.93	3.00	0.09	0.90	0.63
C12:1	0.08	0.08	0.08	0.003	0.85	0.60
C14:0	10.3	10.3	10.3	0.29	0.93	0.95
C14:1 *cis-9*[4]	1.01	1.01	0.98	0.04	0.79	0.55
C14:1 *cis-11*	0.06	0.05	0.05	0.003	0.54	0.39
C16:0	34.7	37.1	37.9	0.52	<0.01	0.15
C16:1 *trans-9*	0.06	0.05	0.05	0.002	0.08	0.90
C16:1 *cis-9*	1.54	1.65	1.60	0.04	0.23	0.06
C16:1 *cis-11*	0.05	0.05	0.04	0.002	0.46	0.22
C16:1 *cis-13*	0.14	0.15	0.14	0.005	0.79	0.43
Total C16	36.7	39.0	39.8	0.53	<0.01	0.17

[1] LP = 3.9 % DM peas

[2] HP = 7.8 % DM peas

[3] SEM = standard error of mean

[4] Data were $\log_{10}$-transformed prior to statistical analysis, but LSM are presented in their original form

3.4.4 RUMINAL FERMENTATION AND PURINE DERIVATIVE INDEX

Ruminal pH, total concentration of VFA, acetate, propionate, and butyrate were not influenced by inclusion of field peas in the diet (Table 9), whereas the concentrations of valerate and BCVFA tended to increase linearly ($P = 0.05$) from 1.39 to 1.57 and from 1.26 to 1.42 mmol/L, respectively. Increasing inclusion rate of field peas in the diet also linearly increased ($P = 0.02$) the ruminal NH_3-N concentration from 6.17 to 8.67 mg/dl. There was no difference in the purine derivative index (PDI) among treatments (Table 10).

Table 9. Effects of increasing rate of pea inclusion in lactating dairy cow diets on ruminal pH, ammonia nitrogen (NH_3-N) and volatile fatty acid (VFA) concentrations

	Treatment				**Significance of contrasts**	
Item	**Control**	**LP[1]**	**HP[2]**	**SEM[3]**	**Linear** ***P-values***	**Quadratic** ***P-values***
pH	6.03	6.10	5.97	0.03	0.47	0.12
Acetate, mmol/L	56.4	56.6	57.0	0.82	0.76	0.96
Propionate[4], mmol/L	17.3	17.6	18.1	0.41	0.49	0.95
Butyrate, mmol/L	12.4	12.9	12.8	0.31	0.54	0.51
Valerate, mmol/L	1.39	1.46	1.57	0.04	0.05	0.79
BCVFA[5], mmol/L	1.26	1.37	1.42	0.03	0.05	0.64
Total VFA[4], mmol/L	88.7	89.9	90.9	1.35	0.62	0.90
Ac/Pr[6]	3.27	3.24	3.20	0.05	0.45	0.98
NH_3-N, mg/dL	6.17	6.50	8.67	0.53	0.02	0.33

[1] LP = 3.9 % DM peas
[2] HP = 7.8 % DM peas
[3] SEM = standard error of mean
[4] Data were $\log_{10}$-transformed prior to statistical analysis, but LSM are presented in their original form
[5] Branched-chain VFA = isobutyric acid + isovaleric acid
[6] Ac/Pr = acetate to propionate ratio

Table 10. Effects of increasing rate of pea inclusion in lactating dairy cow diets on purine derivative index (PDI)

	Treatment				Significance of contrasts	
Item	**Control**	**LP[1]**	**HP[2]**	**SEM[3]**	**Linear** ***P-values***	**Quadratic** ***P-values***
PDI	456.1	463.6	472.3	22.0	0.62	0.17

[1] LP = 3.9 % DM peas

[2] HP = 7.8 % DM peas

[3] SEM = standard error of mean

3.4.5 MICROBIOTA DATA ANALYSES

3.4.5.1 Alpha diversity analysis

On average, 26345 (SD ± 7449) sequencing reads per sample were retained after quality trimming of sequencing data, resulting in an average of 498 (SD ± 84) unique ASVs across samples. The Chao1 (P = 0.98) index of species richness, and Shannon (P = 0.94) and Inverse Simpson (P = 0.74) indices of diversity were not affected by diet (Figure 1).

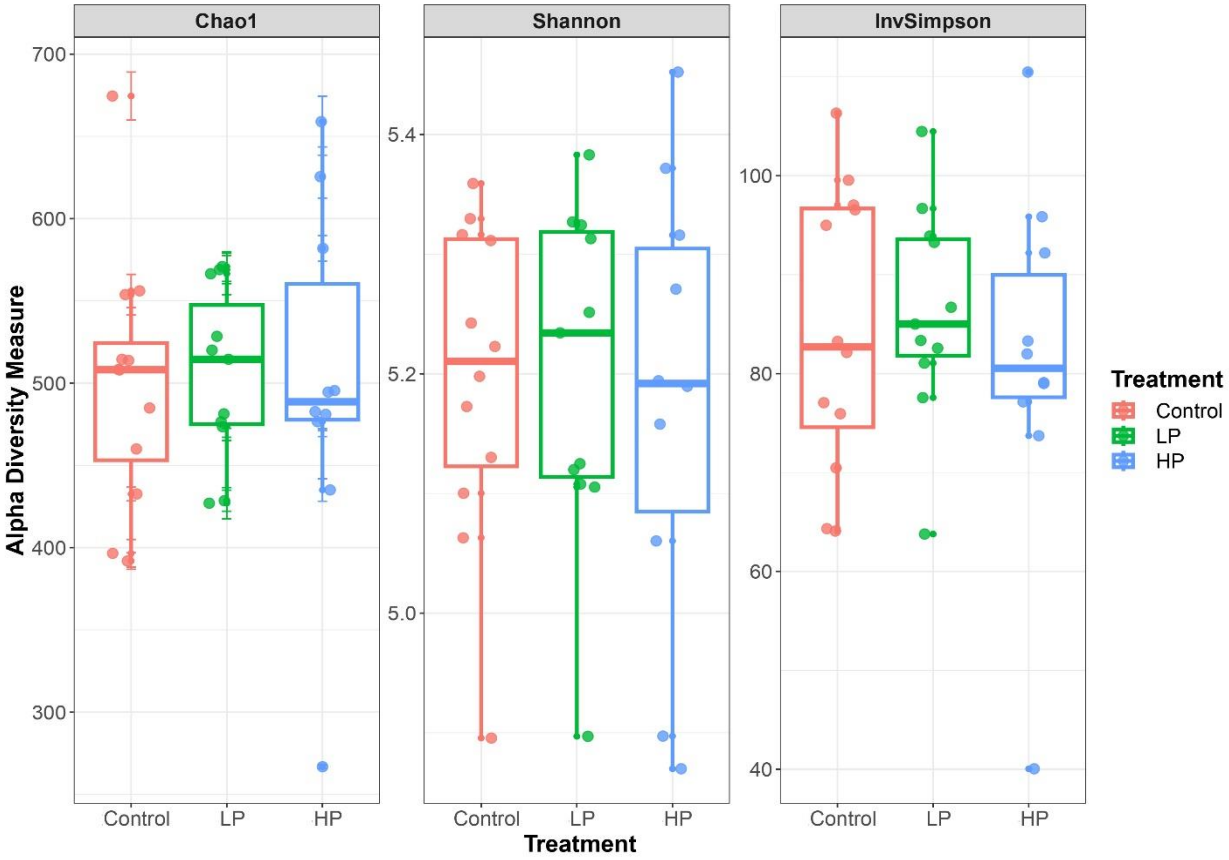

Figure 1. Comparison of the α diversity indices of rumen bacteria among the control, LP, and HP treatments in lactating dairy cows. HP = 7.8 % DM peas group; LP = 3.9 % DM peas group.

3.4.5.2 Beta diversity analysis

The PCA plots (Figure 2A) revealed no clustering patterns among the treatment groups. This observation was supported by PERMANOVA analysis (P = 0.96), which confirmed the lack of significant differences in the composition of rumen bacterial communities across treatments. However, a comparison of the Aitchison's distance among the microbiota of pea-supplemented cows and the control group indicated a trend (P = 0.07) in the composition of communities in response to pea treatment. The rumen bacterial communities of cows in the HP group exhibited higher deviation from the control group compared to those in the LP group (Figure 2B).

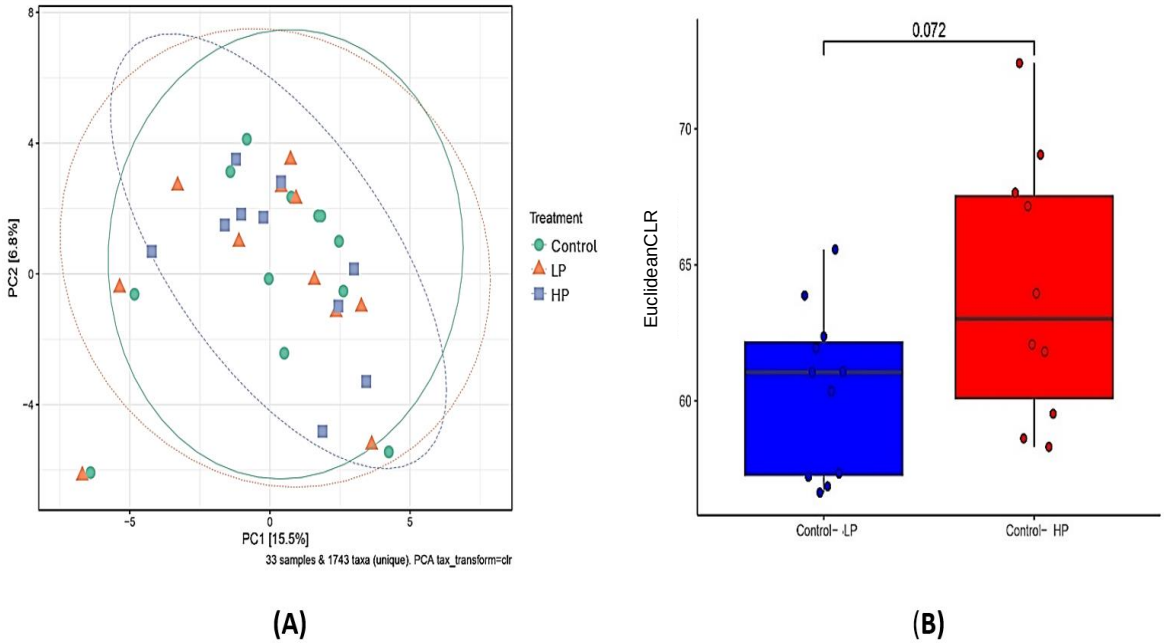

Figure 2. β-diversity analysis of the ruminal bacterial community among treatments in lactating dairy cows (A) Principal Component Analysis (PCA) based on Euclidean distance metrics at the ASV level. (B) Euclidean analysis comparing the ruminal bacterial community between pea-supplemented cows and the control group. HP = 7.8 % DM pea group; LP = 3.9 % DM pea group.

3.4.5.3 Relative abundances of the ruminal bacteria phyla, families, and genera across experimental diets

In total, 16 ruminal bacteria phyla, 72 families, and 157 genera were detected. Irrespective of treatment, the microbiota profile of ruminal samples was dominated by members of Bacteroidota and Bacillota (formerly Firmicutes) phyla, comprising a total of 86.6% of the rumen bacterial community. Prevotellaceae, Lachnospiracaceae, and Ruminococcaceae were the most abundant bacterial families across all treatment groups. *Prevotella*, *Lachnospiraceae* NK3A20, *Succiniclasticum*, and *Ruminococcus* were the most abundant genera among treatments (Supplementary Figures S1 and S2 in Appendix B, and Figure 3, respectively).

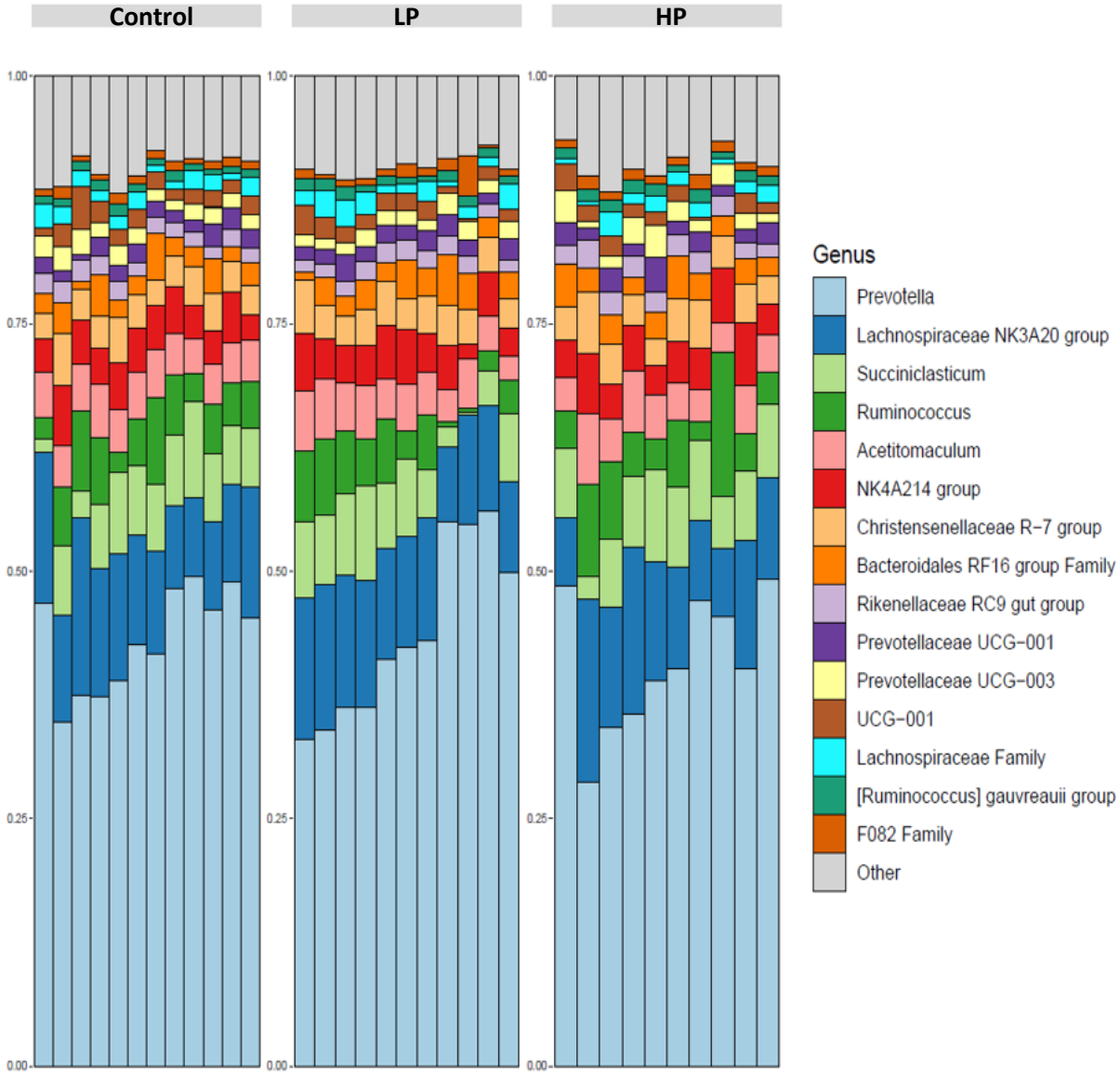

Figure 3. Relative abundances of ruminal bacteria at the genus level across rumen samples in dairy cows. HP = 7.8 % DM peas group; LP = 3.9 % DM peas group.

3.4.5.4 Differential abundance analysis of bacterial taxa among treatment groups at ASV and genus levels

No differences were observed in the abundance of bacterial phyla and families among treatment groups (Supplementary Table S11 in Appendix A). We also did not identify any differences among bacterial genera and ASVs according to Benjamini-Hochberg false discovery rate (BH FDR) adjusted q-values. However, based on p-values, a total of 35 ASVs, including members of Prevotellaceae (ASV35 and ASV48), Lachnospiraceae (ASV50, ASV45, and ASV47), and Ruminococcaceae (ASV30 and ASV24) were found to decrease in response to the inclusion of field peas in the diet ($P < 0.05$). In contrast, 22 ASVs, including members of Prevotellaceae (ASV37, ASV29, and ASV40), Lachnospiraceae (ASV42, ASV43, and ASV55) and Ruminococcaceae (ASV2) were found to increase in response to field pea treatments ($P < 0.05$). The specific members that were decreased or increased differed between the two groups of LP and HP (Figure 4).

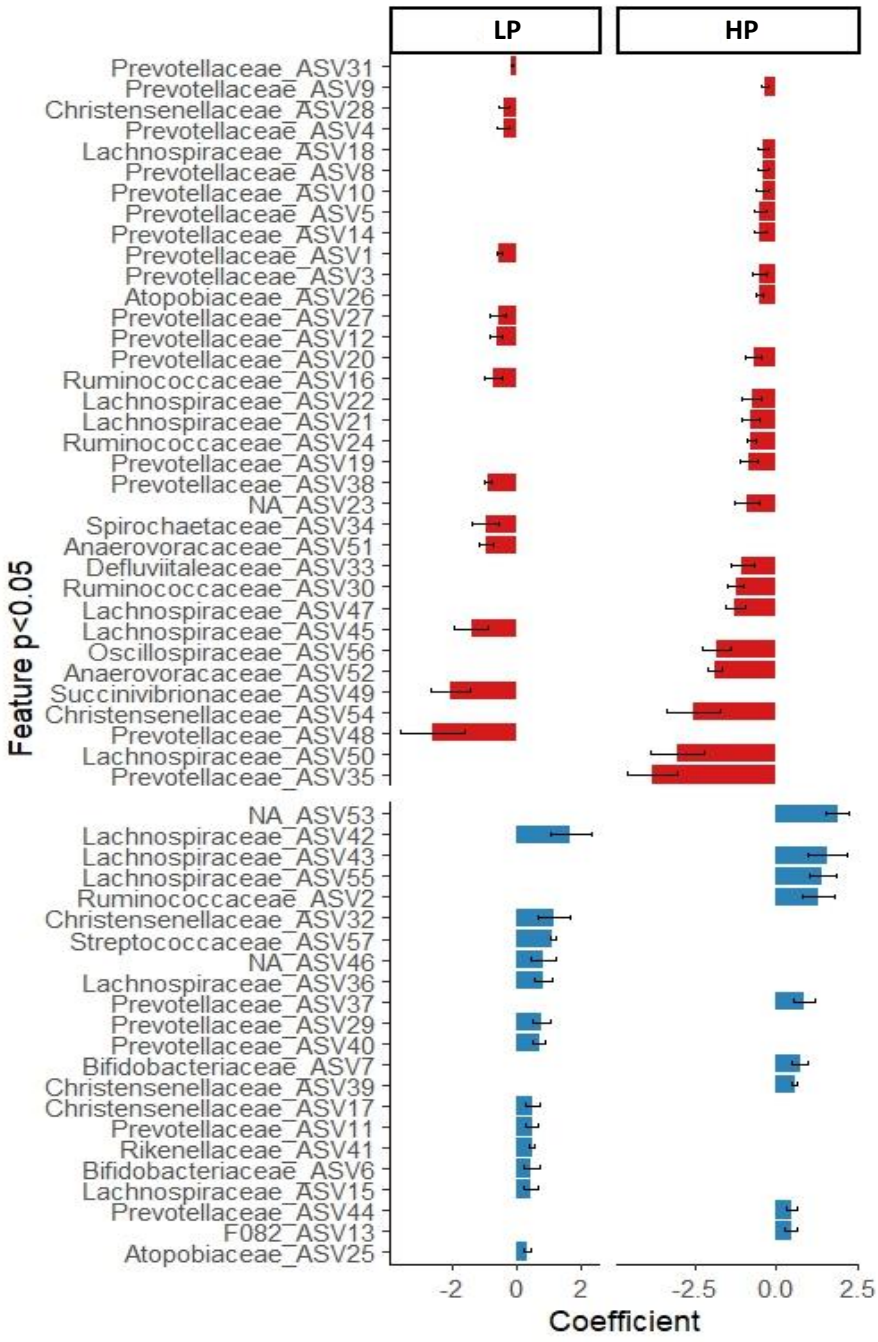

Figure 4. Differential abundance analysis of rumen bacteria at ASV level among treatment groups in dairy cows of LP and HP groups. Blue color shows positive associations, and red color shows negative associations. Differential abundance analysis was performed using CLR transformation and fitting cow, block, and treatment as fixed effect factors in a linear regression model. HP = 7.8 % DM peas group; LP = 3.9 % DM peas group.

At the genus level, the abundance of *Selenomonas* decreased ($P < 0.05$) in response to the inclusion rate of 3.9% DM field peas in the diet. Conversely, the proportions of *Shuttleworthia* ($P < 0.05$) increased in response to the inclusion rate of 7.8% DM field peas in the diet (Figure 5, Supplementary Table S12 in Appendix A).

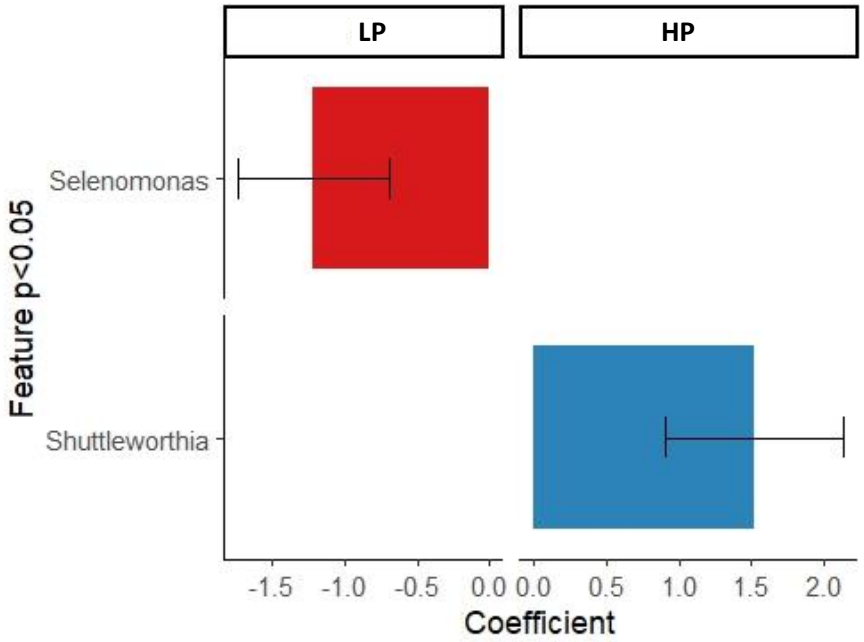

Figure 5. Differential abundance analysis of rumen bacteria at genera level among treatments in dairy cows of LP and HP groups. Blue color shows positive associations, and red color shows negative associations. Differential abundance analysis was performed using CLR transformation and fitting cow, block, and treatment as fixed effect factors in a linear regression model. HP = 7.8 % DM peas group; LP = 3.9 % DM peas group.

3.4.5.5 Spearman correlation of milk and rumen fermentation parameters with the proportion of bacterial genera

Correlation analysis between the proportion of dominant bacterial genera and milk and rumen fermentation parameters revealed that genera *Moryella* (r = - 0.59) and *Marvinbryanti* (r = - 0.62) were negatively correlated with ruminal butyrate concentration ($P < 0.05$), the *Ruminococcus gauvreauii* (r = - 0.57) group exhibited a negative correlation with milk protein content, whereas Olsenella was positively correlated with butyrate (r = 0.53) and rumen NH_3-N (r = 0.52) concentrations ($P < 0.05$; Figure 6 and supplementary Figure S3 in Appendix B).

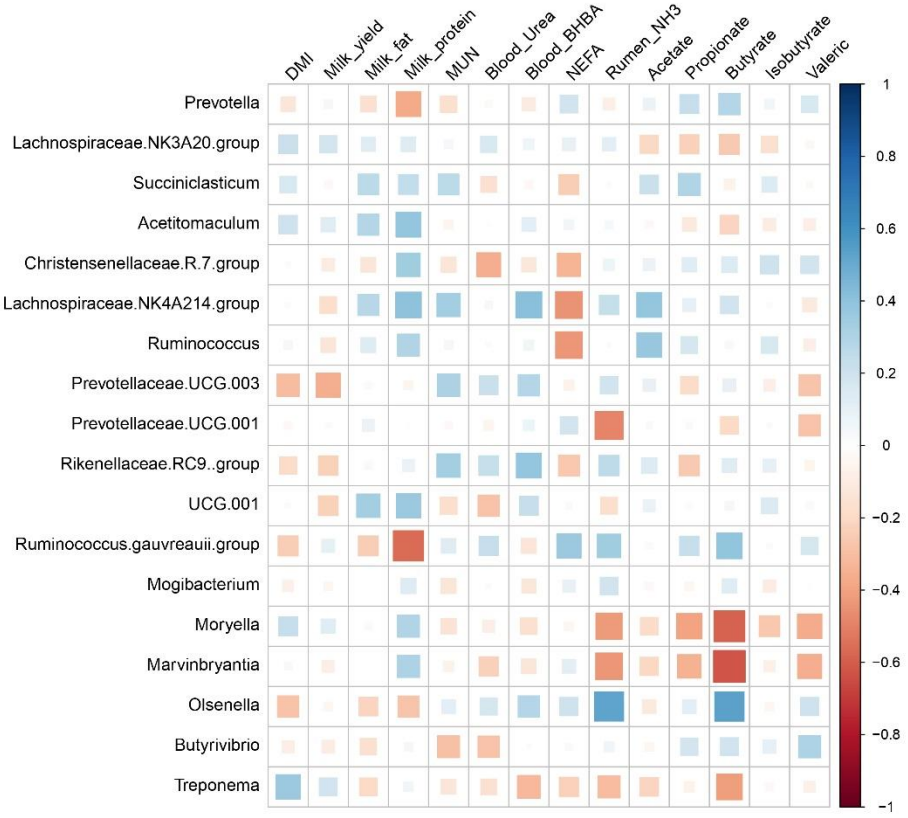

Figure 6. Spearman's rank correlation coefficient of dairy production and rumen fermentation parameters with dominant rumen bacteria genera in dairy cows. Strong correlations are showed by large squares and weak correlations by small squares. Blue color (closer to 1) shows positive correlations, and red color (closer to -1) shows negative correlations. Darker colors indicate higher coefficients.

3.4.5.6 Quantitative real-time PCR analysis

The inclusion of field peas did not affect the copy numbers of total bacterial 16S rRNA gene (P = 0.75), *Prevotella genus* (P = 0.62), *Prevotella bryantii* (P = 0.84), *Ruminococcus genus* (P = 0.77), and *Selenomonas ruminantium* (P = 0.23). Similarly, the ratio of *Prevotella genus* to *Ruminococcus genus* remained unaffected by the inclusion of field peas in the diet (P = 0.80; Figure 7).

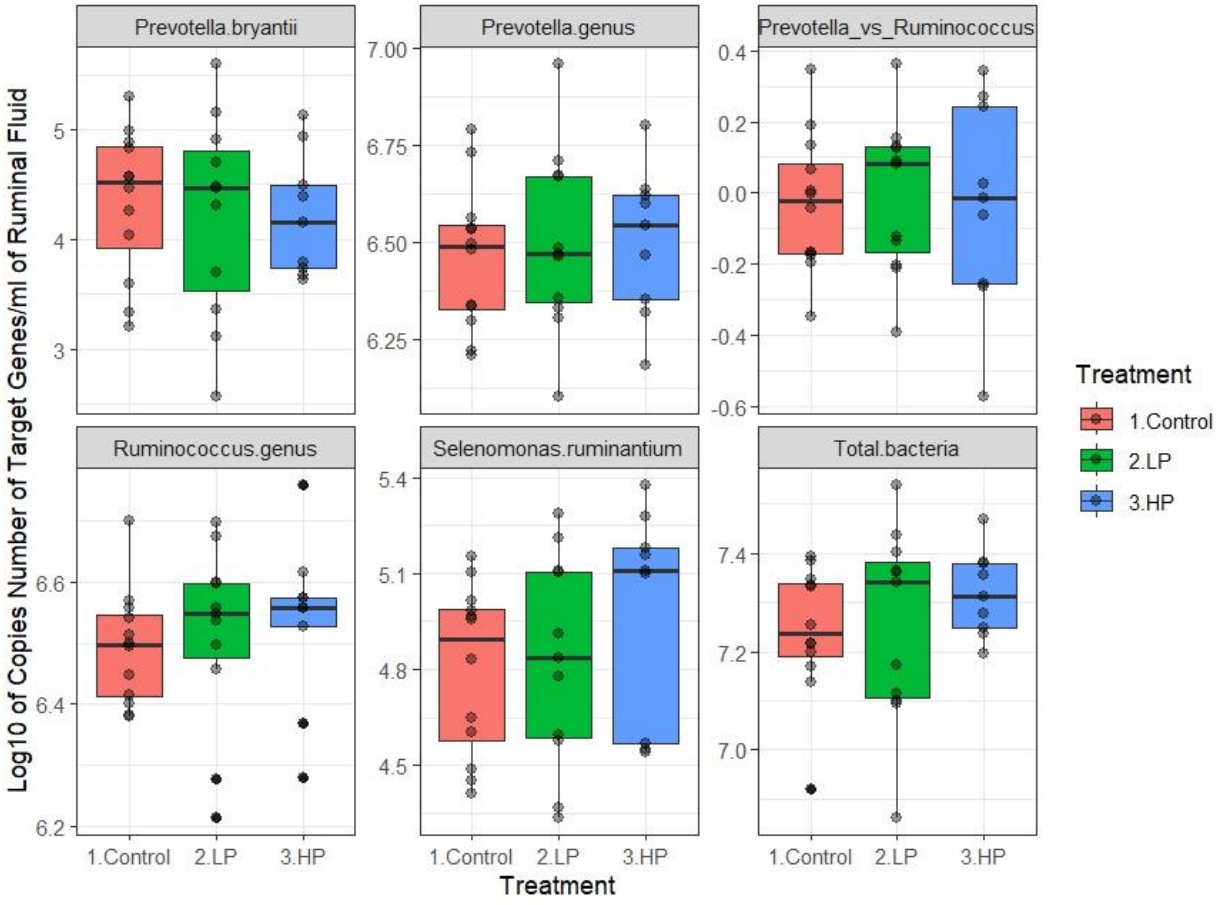

Figure 7. Quantification of total bacterial 16S rRNA genes and selected genera/species of rumen microbiota. Quantitative PCR was performed to measure the copy numbers of bacterial 16S rRNA gene, *Prevotella genus*, *Prevotella bryantii*, *Ruminococcus genus*, and *Selenomonas ruminantium*, as well as the ratio of *Prevotella* genus to *Ruminococcus* genus in lactating dairy cows receiving control, LP (3.9 % DM peas) and HP (7.8 % DM peas) diets.

3.5 DISCUSSION

3.5.1 EFFECTS ON DMI, MILK YIELD AND COMPOSITION

In this study, the increasing inclusion of field peas in lactating dairy cow diets increased DMI quadratically. The increased DMI with the inclusion of field peas in the diet may suggest that field peas can improve the palatability of the experimental diet. Wang et al. (1998) reported that the condensed tannin content in Manitoba-grown field pea cultivars ranged from 0.89 to 5.18 g/kg DM. Therefore, field pea cultivars in Western Canada have low condensed tannin levels, which are unlikely to adversely affect diet palatability. Studies have reported that the inclusion of field peas can enhance diet palatability in young calves (Corbett, 1997; Gilbery et al., 2007). However, due to the differing protein and energy requirements of beef cattle and lactating dairy cows, the findings from these studies are not directly comparable. Conversely, Khorasani et al. (2001) reported that in lactating dairy cows, partially replacing ground barley and fully substituting soybean meal with 10-30% DM field peas in a barley grain-based concentrate diet had no effect on DMI, indicating that pea inclusion did not affect palatability. While the increase in DMI in pea-fed cows in the current study was statistically significant, the actual effect size was small, with the HP group showing only a 0.6 kg/d increase compared to the control. From a practical viewpoint, this improvement in DMI is unlikely to provide meaningful benefits to dairy farmers in terms of milk production and feed efficiency.

In this study, inclusion of field peas in the diet did not affect overall milk yield or protein concentration. The lack of differences in milk protein could be attributed to the relatively similar CP concentration of the isoenergetic experimental diets used. This suggests that the protein's amino acid profile provided by field peas was comparable to that of the control diet ingredients,

supporting similar levels of milk protein synthesis. This also could be attributed to reductions in the ADC of DM, CP, and NDF with the increasing inclusion rates of field peas. Similarly, Petit et al. (1997) reported that partially substituting corn grain and soybean meal with 20.2% extruded or raw peas (DM basis) in a corn grain-based concentrate diet in lactating dairy cows increased DMI without affecting milk yield. However, they observed that extruded peas had higher DMD and nitrogen digestibility, resulting in increased milk protein percentages than those fed raw peas or the control diet. This can be attributed to the increased nutrient digestibility through extrusion, likely due to the reduction of ANFs (Bessada et al., 2019; Petit et al., 1997).

Furthermore, previous studies have concluded that the particle size of field peas affects their nutrient digestibility. Petit et al. (1997) reported that the starch from ground peas tended to be more rumen degradable compared to the starch from cracked peas. Reducing the particle size of peas increases the amount of starch available to rumen microbes (Offner et al., 2003). However, increased VFA production due to higher starch digestibility in the rumen may elevate the risk of subacute ruminal acidosis in dairy cows (Khafipour et al., 2009), which should be taken into consideration. Similar reductions in the digestibility of DM and nitrogen were observed in a study by Vander Pol et al. (2009) when lactating dairy cows were fed 15% rolled peas, but not coarsely ground peas. This was expected to be due to the increased content of undigested pea fragments in feces when rolled peas were used. They also observed a reduction in DMI and milk yield of rolled pea-fed cows, which suggests that rolled peas may not be the most effective processing method for inclusion in dairy cow diets. Therefore, the particle size of field peas was likely not small enough for optimal digestibility in the current study. Similarly, Khorasani et al. (2001) found a decline in the total tract digestibility of NDF in lactating dairy cows with increasing inclusion rate

of 10-30% peas in a barley-based concentrate diet, suggesting that the proportion of digestible fiber is higher in barley compared to peas.

In the current study, increasing the inclusion rate of field peas in the diet of dairy cows resulted in a higher milk fat percentage. Similarly, in a previous study, partially replacing barley grain and canola meal and completely replacing soybean meal and wheat ground with 25% DM field peas in the concentrate (equivalent to 12.5% DM field peas in TMR) in a barley grain-based concentrate diet, increased milk fat percentage of pea-fed cows (Corbett et al., 1995). These authors suggest that the lower degradation rate of pea starch compared to barley starch could potentially increase the acetate-to-propionate ratio, resulting in an increased milk fat percentage. However, in the current study, a corn grain-based concentrate diet was partially replaced with field peas and the degradation rate of corn starch is relatively similar to that of pea starch (McCarthy et al., 1989). In the current study, ruminal acetate concentration did not differ among treatments; therefore, the increase in milk fat percentage cannot be attributed to changes in rumen microbial fermentation. However, the crude fat content of the experimental diet in the high-pea group was slightly higher than that of the control group, which may partially explain the observed changes in milk fat content.

3.5.2 EFFECTS ON RUMEN FERMENTATION AND RUMEN BACTERIAL COMMUNITY COMPOSITION

In this study, the pH and proportions of acetate, propionate, butyrate, and total VFA in the rumen were not affected by diet. In contrast, Khorasani et al. (2001) reported that gradually replacing ground barley and soybean meal with 10-30% DM peas in lactating dairy cow diets

decreased ruminal pH and increased total VFA concentration. In the current study, field peas were incorporated into a corn-based concentrate diet, and the degradation rate of pea starch is comparable to that of corn starch (Cerneau and Michalet-Doreau, 1991), which may explain the absence of changes in ruminal pH and VFA concentrations in response to the diet. In contrast, in the study by Khorasani et al. (2001) study, field peas were included in a barley grain-based concentrate diet, where barley starch has a higher rumen degradability than pea and corn starch (McCarthy et al., 1989), leading to greater VFA production and a lower rumen pH due to the increased microbial fermentation of the more degradable starch in Khorasani et al. (2001) study. Additionally, the higher pea inclusion rates and the replacement of only soybean meal as a main protein source in the diet with field peas in the Khorasani et al. (2001) study likely led to a greater increase in dietary starch content compared to this study. Another study reported that partially substituting corn grain and soybean meal with 15% DM of coarsely ground peas in lactating dairy cow diets did not influence ruminal pH and total VFA (Vander Pol et al., 2009). These discrepancies between studies may be attributed to differences in diet composition, formulation, and the specific ingredients replaced by field peas.

In the current study, the concentrations of valeric acid and total BCVFA (isobutyrate and isovalerate) increased with increasing inclusion rates of field peas, which aligns with the findings of Khorasani et al. (2001), suggesting an increase in microbial protein degradation. Additionally, in this study, increasing the dietary contents of field peas increased the concentrations of rumen NH_3-N, blood urea, and MUN. These changes indicate increased protein degradation and deamination of AA in the rumen, suggesting that pea protein has higher rumen degradability compared to corn and soybean meal protein (Khorasani et al., 2001; Mitchell et al., 2023; Vander Pol et al., 2009). Although the inclusion of field peas in the diet linearly increased ruminal NH3-

N concentration due to higher RDP intake, microbial protein synthesis was not affected. McCarthy et al. (1989) reported that when protein degradation exceeds the available carbohydrate supply as an energy source in the rumen, microbial nitrogen capture efficiency decreases. This outcome is further supported by a study demonstrating that replacing ground barley and soybean meal with 10-30% DM field peas in a barley grain-based concentrate diet led to increased milk protein concentration in pea-fed cows only at lower inclusion rates.

Reducing the RDP concentration of field peas through processing methods such as extrusion, which in turn reduces the concentration of rumen NH_3-N in cows receiving field peas, has been reported to increase the supply of MCP in their small intestines (Focant et al., 1990; Walhain et al., 1992). In this study, the excretion of allantoin, creatinine, and uric acid in urine was measured to calculate PDI, which serves as a marker of microbial protein synthesis (Makkar and Chen, 2004), and no differences in PDI were found among treatments. This suggests that the inclusion of field peas did not impact ruminal microbial protein synthesis. Additionally, no differences were observed in the total 16S rRNA gene abundance per mL of rumen fluid among treatment groups, suggesting that overall bacterial biomass was not affected by inclusion of field peas in the diet. Similarly, in a study by Vander Pol et al. (2008) when 15% DM peas partially replaced corn and soybean meal in a corn grain-based concentrate diet of lactating dairy cows, it did not affect the urinary excretion of allantoin and uric acid, suggesting a comparable passage of microbial protein from the rumen among treatments.

As inclusion rate of field peas in lactating dairy cows was expected to increase the rumen concentration of NH_3-N and deamination of AA, we hypothesized that partially substituting a corn-based concentrate diet with field peas affects both the biomass and composition of the rumen bacterial population, specifically increasing the abundances of proteolytic bacteria in the rumen.

Although the inclusion of field peas in the diets of lactating dairy cows did not influence alpha diversity indices, the beta-diversity comparison among treatment groups revealed a shift in the overall composition of the bacterial communities in the rumen of cows receiving pea diets compared to the control. Studies have shown that the rumen microbial community is dynamic, and when animals experience abrupt shifts in their diet, the rumen microbiome undergoes alterations in its microbial community structure in response to the changes in diet composition (Chen et al., 2021; Weimer, 2015).

Consistent with previous studies of the rumen microbiota in lactating cows, Bacteroidota and Bacillota (formerly Firmicutes) were the most abundant rumen bacterial phyla in this study (Castillo-Lopez et al., 2018; Derakhshani et al., 2017). The predominant bacterial families included Prevotellaceae, Lachnospiraceae, and Ruminococcaceae (Castillo-Lopez et al., 2018). The inclusion of field peas up to 7.8% DM did not affect the relative abundances of any of the major bacterial phyla and families. These results are consistent with the findings of Castillo-Lopez et al. (2014), who reported that increasing the substitution of corn grain with dried distillers grains with solubles (DDGS), a high-protein feed ingredient, while maintaining relatively similar CP content across all diets, had no impact on the most abundant rumen bacterial phyla and genera, including *Prevotella*, *Succiniclasticum*, and *Ruminococcus*. In this study, we also observed no changes in the proportion of dominant bacterial genera, including *Prevotella* and *Ruminococcus*. However, higher resolution differential abundance analyses suggested changes in the proportions of several ASVs belonging to Prevotellaceae, Lachnospiraceae, and Ruminococcaceae in response to the pea inclusion rate. This finding may reflect the species- or strain-level response variation to the treatment diets (LP and HP) within these bacterial lineages.

Previous studies using both traditional cultivation methods (Gylswyk, 1990) and 16S rRNA gene amplicon sequencing method (Henderson et al., 2015) have identified *Prevotella* genus as the dominant rumen bacteria. The genus *Prevotella* of the phylum Bacteroidota has the ability to utilize a broad range of nutrients, such as proteins, starch, sugar, pectin, and cellulose (Cholewińska et al., 2020; Fievez et al., 2012; Xue et al., 2019). A previous study identified *Prevotella ruminicola*, *Prevotella bryantii*, *Prevotella brevis*, and *Prevotella albensis* as the dominant *Prevotella* species in the rumen, playing a crucial role in the degradation of plant polysaccharides (Kim et al., 2011). However, different *Prevotella* strains exhibit varying substrate utilization capabilities. For example, BP1-145/148-like strains primarily utilize xylan and pectins, while some strains, such as AGR2160, cannot utilize xylan but can degrade other hemicelluloses. In contrast, *Prevotella brevis* and *Prevotella stercorea* lack the ability to degrade hemicellulose (Accetto and Avguštin, 2019). In the current study, the concentrations of CP were approximately similar across all experimental diets and according to qPCR results, the abundance of *Prevotella genus* was similar across treatment groups. However, differences in the proportion of several ASVs belonging to Prevotellaceae, observed in 16S rRNA gene sequencing analyses, may reflect inter- and intra-species differential responses within this bacterial lineage to inclusion of peas in the diet. Further metagenomic studies are needed to distinguish species- and strain-level dynamics within the rumen microbiome with an increasing inclusion rate of peas in lactating dairy cows. Although we observed increased ruminal NH_3-N concentration with increasing inclusion rates of peas, this may not have affected the rumen bacterial community composition as NH_3-N was not limiting for microbial protein synthesis in any of the treatment groups compared to studies that have evaluated diets with varying concentrations of CP.

In a study by Kim et al. (2011), members of Ruminococcaceae and Lachnospiraceae were among the most prevalent bacterial families in the rumen within the Clostridia class of the Bacillota phylum, representing 25.8% and 23.8% of Clostridia-related reads, respectively. The real-time qPCR results demonstrated that the abundance of *Ruminococcus genus* did not differ among treatments. *Ruminococcus* species are among the main fibrolytic bacteria in the rumen, efficiently degrading plant fibers (Krause et al., 1999). However, *Butyrivibrio fibrisolvens*, another major fibrolytic bacterium in the rumen, grows more rapidly on soluble forage components, suggesting that differences in substrate solubility influence bacterial competition (Kalmokoff and Teather, 1997). A previous study stated that Lachnospiraceae accounted for up to 40% of the total bacterial community, demonstrating their abundance in the rumen (Kittelmann et al., 2013). Additionally, Lachnospiraceae members are known for butyric acid production, but the functional diversity of this family within the rumen ecosystem remains largely unexplored (Meehan and Beiko, 2014). While the proportion of *Ruminococcus* remained similar across treatment groups in qPCR results, 16S rRNA gene sequencing suggested that several ASVs within the Ruminococcaceae and Lachnospiraceae families exhibited changes in response to the inclusion of field peas. This further suggests that shifts in the composition and function of the rumen microbiota are species- and strain-specific, highlighting the need for higher resolution compositional and functional analyses of the rumen microbiome using metagenomics and/or metatranscriptomic approaches.

Unsaturated fatty acids in the rumen are primarily converted to saturated fatty acids by rumen bacteria (lipolytic species) through the biohydrogenation process (Jenkins et al., 2008), affecting the composition of fatty acids in milk (Carreño et al., 2019). Members of genera *Pseudobutyrivibrio* (Paillard et al., 2007), *Butyrivibrio* (Polan et al., 1964), *Propionibacterium* (McKain et al., 2010), and *Selenomonas* (Fujimoto et al., 1993) in the rumen have been identified

as being involved in biohydrogenation activity. In this study, qPCR analysis indicated that the diet had no effect on the abundance of *Selenomonas ruminantium* in the rumen of dairy cows. However, the differential abundance analysis using 16S rRNA gene amplicon sequencing demonstrated that the abundance of the genus *Selenomonas* decreased in response to the inclusion rate of 3.9% DM field pea. This discrepancy may be attributed to species-level response variations within the *Selenomonas* genus to the treatment (Mattila et al., 1988). An *in vitro* study using 0.6 and 1.0 mg of tannin/mL of ruminal fluid reported that dietary tannins can reduce the activity of bacteria responsible for biohydrogenation, potentially affecting rumen bacterial populations (Vasta et al., 2008). Field peas contain ANFs such as tannins (Bessada et al., 2019); however, the proportion of PUFA decreased with increasing the inclusion rate of field peas in this study, suggesting that tannin did not influence the biohydrogenation of PUFA in the rumen. As stated by Goel et al. (2005), the rumen of cattle contains tannin-tolerant bacteria, such as *Selenomonas ruminantium* that produce tannase, enabling the cow to convert tannins into acetate and butyrate. Palmitic acid (C16:0), oleic acid (C18:1 cis-9), and linoleic acid (C18:2 n6) were the main fatty acids in the field peas used in this study. The elevated proportion of C16:0 observed in the milk of pea-fed cows may be due to the higher proportion of palmitic acid in field peas experimental diets, rather than increased biohydrogenation. In this study, soybean meal, corn grain, and canola meal were partially replaced with field peas. Soybean meal, corn grain, and canola meal contain higher proportions of C18:1 and C18:2 fatty acids than peas (Barrera-Arellano et al., 2019; Lewinska et al., 2015; Reveglia et al., 2025). The decreased proportion of C18:1 and C18:2 fatty acids observed in the milk of pea-fed cows could be attributed to the lower levels of these fatty acids in field peas. Additionally, the elevated proportion of C16:0 and decreased levels of C18:1 and C18:2 in the experimental pea

diets, in comparison to the control diet, may further explain the changes observed in the milk fatty acid profile.

3.6 CONCLUSIONS

This study confirmed our hypothesis that partially replacing field peas in a corn grain-based concentrate diet would increase rumen NH_3-N, MUN, and blood urea concentrations due to an increase in the degradation of dietary protein in the rumen. Additionally, we observed elevated concentration of BCVFA in pea-fed cows, indicating enhanced deamination of branched-chain amino acids in the rumen. While increasing the inclusion rate of field peas in lactating dairy cow diets increased MUN concentration, the diet did not significantly affect milk production and microbial protein synthesis in the rumen. Increasing the field pea inclusion rate improved DMI and milk fat percentage, although it negatively affected the digestibility of DM, CP, and NDF while having no impact on milk protein content. This study demonstrated that incorporating field peas into lactating dairy cow diets at up to 7.8% of DM did not change the overall composition of the rumen bacterial community, as long as the diets remained isoenergetic and had comparable CP content. Total bacterial abundance remained consistent across treatments, indicating that overall microbial biomass and microbial protein synthesis were unaffected by diet. The lack of impact on PDI further supports the absence of dietary effects on microbial protein synthesis. Overall, findings of this study suggest that field peas can be replaced in a corn grain-based concentrate diet of lactating dairy cows at inclusion rates of up to 7.8% without negative effects on milk yield, milk composition, feed intake, rumen fermentation, and dominant rumen bacterial taxa. Despite increases in rumen NH_3-N, blood urea, and MUN concentrations, as well as shifts in milk fatty

acid composition, including a higher level of C16:0 and reductions in C18:2 isomers, production performance and rumen fermentation remained stable with field pea inclusion rate in the diet. Further research is needed to comprehensively assess changes in the dynamics and functionality of the rumen microbiome in response to field pea inclusion rates by integrating 16S rRNA gene amplicon sequencing with complementary approaches, such as metagenomics, metatranscriptomics, or metaproteomics. Particularly, the use of parallel multiomics approaches such as metatranscriptomics and metaproteomics can provide information on the shifts in the expression of metabolic pathways of the rumen microbiome in response to dietary intervention.

CHAPTER 4: GENERAL DISCUSSION AND CONCLUSIONS

4.1 GENERAL DISCUSSION

Soybean meal and canola meal are the primary sources of protein in lactating dairy cow diets, while corn and barley meals are the main sources of starch (Masoero et al., 2006). However, due to fluctuations in their cost and availability (Jezierny et al., 2010), there is increasing interest in the production of grain legumes, such as field peas, as an alternative to traditional protein and energy source for animal nutrition. Peas are a beneficial rotational crop for cereal production systems (Vander Pol et al., 2008) and they help improve soil fertility (Courty et al., 2015). Feed costs make up a significant portion (40-60%) of total expenses on dairy farms. Therefore, optimizing feeding strategies plays an essential role in enhancing the economic sustainability and profitability of dairy farming (Finneran et al., 2012). However, contradictory outcomes have been reported in the literature regarding the inclusion of peas into lactating dairy cow diets, and to our knowledge, there is only one study evaluating the impact of inclusion of peas on rumen microbiota composition and structure. In the current study, field peas were provided to lactating dairy cows to investigate the impact of partial substituting corn grain-based consecrate diet with 3.9% and 7.8% DM field peas on milk yield and composition, DMI, rumen fermentation parameters, blood metabolites, and rumen bacterial community composition.

While field peas are high in RDP concentration, it may limit how much can be included in the diet of lactating dairy cows. Inclusion of peas in dairy cow diets may reduce milk yield, and especially milk protein yield, in high-yielding dairy cows, when the demands for RUP content are not met and the metabolizable protein availability is insufficient to support their production requirements (Corbett et al., 1995). In our study, all experimental diets were formulated to contain

several high-protein feeds, including canola meal, soybean meal, porcine meat meal, and distiller's grain. This followed the recommendation of Petit et al. (1997) that when peas are used as a protein supplement in the diet of high-producing lactating cows, they should not be the only source of dietary protein, as they may not provide sufficient RUP to meet the higher protein demands of these cows and could result in excessive production of rumen NH_3-N, blood urea, and MUN. Additionally, when incorporating peas into the diet of dairy cows, it is important to ensure correct amino acid balance of metabolizable protein, as peas contain less methionine than soybean and corn meals (Masoero et al., 2006). As a result, the highest inclusion rate of field peas in our study was 7.8% DM. This inclusion rate was lower than what was reported in earlier research (Corbett et al., 1995; Khorasani et al., 2001; Pereira et al., 2017; Petit et al., 1997; Vander Pol et al., 2008), but is comparable to the 10.3% DM reported by Masoero et al. (2006).

In the current study, no significant effect was observed in milk yield and milk protein when 3.9% or 7.8% of raw peas were substituted as an alternative protein and energy source in a corn grain-based concentrate diet. These findings align with those reported by Masoero et al. (2006), where partially replacing soybean meal and barley with raw peas at an inclusion rate of 10.30% DM had no impact on milk yield and composition. However, we observed that inclusion of field peas in the dietary intake of dairy cows increased DMI and milk fat percentage while having no impact on milk yield and milk protein concentration. This outcome might be attributed to the observed reductions in the apparent total tract digestibility of nitrogen resulting from the incorporation of peas. Petit et al. (1997) found that inclusion of 20.2% raw peas (DM basis) with soybean meal and corn grain in a corn grain-based concentrate diet resulted in increased DMI without impacting nitrogen digestibility for the cows consuming the raw peas, which in turn did not affect milk yield or composition. However, substituting soybean meal with extruded peas

increased the digestibility of DM and nitrogen for the cows consuming the extruded peas. Consequently, these cows tended to produce more milk protein as their DMI increased, likely due to improved nitrogen digestibility, which provided more nitrogen for synthesizing milk protein. Petit et al. (1997) found that extruding peas enhanced the ruminal degradability of starch without affecting the ruminal degradable protein. However, Masoero et al. (2005) reported that extruding peas decreased the content of RDP while increasing the ruminal degradability of starch. The findings indicate a potential factor contributing to increased microbial protein synthesis and increased nitrogen availability for milk protein.

In this study, the increasing inclusion of field peas led to a linear increase in the concentration of MUN. Elevated MUN could be indicative that the diet of lactating dairy cows contains excessive RDP or insufficient rumen available energy for microbial protein synthesis. This imbalance can lead to excess ruminal NH_3-N, increased urea excretion in urine, and consequently, reduced nitrogen utilization efficiency along with energy and AA losses (Jonker et al., 1998; Mutsvangwa et al., 2016; Reed et al., 2017). Recommended optimal MUN concentration in dairy cows ranges from 10 to 16 mg/dl (Jonker et al., 1998). Hence, the MUN concentration of cows on the HP diet in this study was still within the recommended range, implying that the inclusion of field peas up to 7.8% of DM would not negatively affect milk quality and protein metabolism in the rumen.

The fatty acid composition of the diets differed across treatments in this study. For example, the C16:0 level was higher in the LP and HP groups (45.3 and 49.0 g/100 g of total fatty acids, respectively) compared to the control group (43.8 g/100 g of total fatty acids). Consequently, the observed linear increase in the yield of C16:0 fatty acid in milk, following the inclusion of field peas in the diet is likely attributed to the higher proportion of C16:0 fatty acid in the pea

experimental diets, rather than an increase in the biohydrogenation process. Additionally, the differences in the fatty acid composition, specifically the proportions of oleic, linolenic, and linoleic acids among corn, soybean meal, and peas may be responsible for changes observed in the fatty acid composition of milk in dairy cows when field peas replace corn and soybean meal in their diet (Barrera-Arellano et al., 2019; Lewinska et al., 2015; Villalobos Solis et al., 2013).

The composition of the rumen bacterial community is influenced by different factors, with diet being one of the major determinant (Henderson et al., 2015). Therefore, understanding the response of ruminal microbiota to various inclusion rates of field peas as an alternative to traditional dairy supplements, such as soybean meal, corn grain, canola meal, and barley grain enables us to better understand microbial mechanisms underlying changes in rumen fermentation, particularly markers of protein metabolism and VFA. The VFA, primarily acetate, propionate, and butyrate, are key products of rumen microbial fermentation which are absorbed by the rumen wall and contribute up to 70% of the energy supply for ruminants (Bergman, 1990). In this study, the inclusion of field peas in the dietary intake of dairy cows did not affect the total or individual concentrations of VFA in the rumen. This is consistent with Vander Pol et al. (2009) where partially replacing corn grain and soybean meal with 15% of field peas (DM basis) in a corn grain-based concentrate diet had no impact on acetate, propionate, butyrate, and total VFAs in the rumen. In contrast, partially replacing barely and completely replacing soybean meal with 10-30% field peas in a barley grain-based concentrate diet by Khorasani et al. (2001) increased the total concentration of VFA and changed the rumen fermentation profile. The reason for this discrepancy may be due to variations in diet ingredient composition, the specific ingredients replaced with peas, the inclusion rate of peas, and the stage of lactation of the cows.

Rumen microbial fermentation plays a vital role in microbial protein synthesis, supplying the majority of the metabolizable protein that reaches and is absorbed through the small intestine of ruminants (Schwab and Broderick, 2017). Rumen-degradable proteins are degraded by proteolytic bacteria into their constituent AA, which are then deaminated to release ammonia, the primary nitrogen source for rumen bacteria to synthesize MCP (Bach et al., 2005; Keum et al., 2024). In this study, increasing the inclusion rate of field peas significantly elevated the concentrations of rumen NH_3-N and BCVFA. This increase indicates enhanced protein break down and AA deamination in the rumen, as a result of the high RDP concentration in field peas. Satter and Slyter. (1974) identified that to maximize the growth of rumen bacteria, ruminal NH_3-N concentration must exceed 5 mg/dl. We also found that the ruminal NH_3-N concentration increased from 6.17 mg/dl to 8.67mg/dl with increasing the inclusion rate of peas. Additionally, research conducted by Khorasani et al. (2001) revealed that incrementally substituting ground barley and soybean meal with 10-30% DM of peas led to a linear increase in ruminal NH_3-N concentration, rising from 12.4 mg/dl in the no-pea group to 15.9 mg/dl when soybean meal was completely replaced by peas. The higher solubility and rumen degradability of the CP in peas than in soybean meal and corn may explain this increase. Consequently, the higher degradation of CP and deamination of AA in the rumen result in elevated concentrations of ruminal NH_3-N. However, when excessive amounts of ruminal NH_3-N are produced, they enter the bloodstream, are converted to urea in the liver, and are excreted in urine, contributing to environmental pollution (Savari et al., 2018). Therefore, the inclusion rate of peas in the diet of lactating dairy cows should not be high to prevent excessive NH_3-N production. According to the study by Masoero et al. (2006), when soybean meal and barley were partially replaced with 10.3% raw peas, the ruminal NH_3-N concentration was observed to be higher than that of extruded peas as well as the control

group (17.8 mg/dl for raw peas compared to 13.6 mg/dl for extruded peas and 14.2 mg/dl for the control group). This suggests that decreasing the RDP content by extruding peas may lead to lower ruminal NH_3-N concentration. However, the absence of RUP, RDP, and MCP measurements in this study is a limitation, as it prevents an accurate evaluation of rumen protein metabolism and its response to the treatment.

In this study, PDI, an indicator of microbial protein synthesis, was not influenced by the diet. Synchronizing sufficient energy with nitrogen supply in the rumen is necessary to increase nitrogen utilization efficiency (Nocek and Russell, 1988). Since starch ferments faster than NDF in the rumen, energy derived from starch fermentation can enhance rumen microbial activity and MCP synthesis (Hall and Huntington, 2008). All experimental diets in the current study had relatively similar CP and starch contents. Although the RDP content of peas is higher than that in corn, the comparable rumen starch degradation rates of peas and corn likely explain the similar PDI observed when peas was partially substituted in the diet.

Increasing inclusion rates of field peas also did not affect the relative abundances of predominant rumen bacterial phyla and families. This finding aligns with the results of Castillo-Lopez et al. (2018), where substituting a barley-based concentrate diet with 11.4% flaxseed-based supplements, which included 37.8% field peas (equivalent to 4.31% in TMR on a DM basis), had no significant impact on the overall rumen microbiota composition in dairy cow diets. Similarly, qPCR results did not show significant differences in the abundance of the *Prevotella* and *Ruminococcus* genera. However, high-resolution differential abundance analyses at the ASV level revealed shifts (increases and decreases) in the proportions of several ASVs belonging to Prevotellaceae, Lachnospiraceae, and Ruminococcaceae in response to the inclusion of field peas, suggesting species- and strain-level diversity within these groups of bacteria (Accetto and

Avguštin, 2019; Krause et al., 1999). Marker-based amplicon sequencing, such as the 16S rRNA gene, and metagenomic shotgun sequencing have been widely utilized in the literature to study rumen microbial composition (Henderson et al., 2015; Stewart et al., 2019). In the current study, we performed sequencing of partial 16S rRNA gene to assess changes in rumen microbiota composition in response to two different inclusion rates of field peas in the diet of lactating dairy cows, which has limitations in distinguishing species- and strain-level diversity in complex microbial communities, we cannot draw definitive conclusions about taxonomic inconsistencies in response to the diet or the functional potential of these taxa. Due to the complexity of the rumen bacterial community, a comprehensive analysis of bacterial changes in response to diet requires integrating 16S rRNA gene sequencing with complementary approaches such as metagenomics, metatranscriptomics, or metaproteomics (Hart et al., 2018; F. Li and Guan, 2017) to better capture changes in the dynamics of rumen microbiome and its functionality in response to the inclusion of field peas. The discovery of novel species through metagenomics and cultivation efforts expands our understanding of the rumen bacterial community. In previous studies, many uncultivated new species and strains of rumen bacteria, such as *Prevotella* were identified (Stewart et al., 2019), and many of them were successfully cultured and isolated (Grabner et al., 2023). According to previous studies, the rumen microbiota, including the polysaccharide-degrading bacterial community, exhibits functional redundancy, indicating that multiple species can perform similar roles in breaking down complex plant polysaccharides (Weimer, 2015). Additionally, while competition among microbes influences which species dominate, niche specialization also plays a key role (Martens et al., 2014). Although qPCR results indicated that the abundance of *Prevotella* and *Ruminococcus* genera remained consistent across treatments, we cannot draw a definitive conclusion about the lack of responses in fibrolytic groups due to the sampling technique used for

collecting rumen fluid. In this study, rumen fluid samples were collected via rumenocentesis, which may not fully capture the overall rumen bacterial composition, as it tends to under-represent fiber-associated microbial communities concentrated in the solid fraction of the rumen. This study highlights the potential for field peas to be included in the diet of lactating dairy cows as a locally sourced, protein-rich alternative. However, it is important to carefully manage the inclusion rate of field peas to ensure optimal benefits while avoiding amino acid loss and negative environmental impacts through excessive ammonia production.

4.2 GENERAL CONCLUSIONS

Field peas are a good source of CP, with high rumen degradability in lactating dairy cows, and this study confirmed these characteristics, as evidenced by increased concentrations of ruminal NH_3-N, BCVFA, blood urea, and MUN with increasing inclusion rates of peas in the diet. Therefore, diets should be formulated with sufficient RUP to bypass rumen degradation and meet the essential AA requirements of lactating dairy cows. This is why the inclusion rate of field peas should not be too high, as their high rumen degradability may not provide sufficient RUP to meet the needs of high-yielding lactating dairy cows. This study showed that field peas can serve as an alternative to traditional protein sources in lactating dairy cow diets at an inclusion rate of up to 7.8%. With the increasing cultivation and availability of field peas in Canada, particularly in Western Canada, over the past decade, the dairy sector can benefit from a locally sourced, protein-rich feed ingredient. In this study, an increase in DMI and milk fat percentage in pea-fed cows did not affect overall milk yield or protein content. We recommend using finely ground field peas, with a smaller particle size than those used in this study, to improve nutrient digestibility. In this

study, PDI and total bacterial abundance were unaffected by diet, indicating similar overall microbial biomass and subsequent microbial protein bypass from the rumen among treatments. Balancing dietary RDP concentration with the cows' nutritional needs, while considering factors such as lactation stage and diet composition, is essential for improving milk production and sustainability in the dairy sector. The results indicated that diets based on corn grain-based concentrate can be partially replaced by field peas in lactating dairy cows at an inclusion rate of up to 7.8% without adversely impacting milk yield, milk protein, milk fat, feed intake, VFA, and rumen bacterial community composition. Further research into processing methods, such as extrusion, and their effects on protein utilization could provide insights into increasing the efficiency of field peas in dairy nutrition.

4.2.1 LIMITATIONS AND FUTURE RESEARCH

The Latin square design is efficient for studies on cattle nutrition and allows for replication with a limited number of animals (Kononoff and Hanford, 2006). However, the presence of carryover effects can influence the results (Castillo-Lopez et al., 2018). To address this limitation, a 21-d period was established for the experiment. The initial 2 weeks were designated as the adaptation period, and the final week was reserved for sampling. Furthermore, in this study, rumen fluid samples were collected via rumenocentesis, which may not provide a fully comprehensive representation of the whole rumen bacteria composition, as it tends to under-represent the fiber-associated bacterial community and more frequent rumen samples are needed.

Future studies should not only examine the composition of the rumen bacterial community following the inclusion of field peas in the diet of lactating dairy cows but also investigate the

functional potential and metabolic aspects of genes within the rumen microbial community using a multi-omics approach, combining the powers of metagenomics, metatranscriptomics, and metabolomics. Additionally, previous studies have shown that extrusion positively influences the reduction of the RDP concentration in field peas, enhances nutrient digestibility, and increases milk protein concentration in lactating dairy cows (Masoero et al., 2006; Petit et al., 1997). Therefore, further studies are needed to examine the impacts of different inclusion rates of extruded field peas on rumen fermentation, as well as the composition and functionality of the rumen bacterial community in lactating dairy cattle. Furthermore, the high concentration of phenolic compounds in leguminous seeds, such as field peas provides antioxidant properties that can affect milk flavor (Duenas et al., 2004). Therefore, further research is needed to assess the impact of including field peas in lactating dairy cow diets on milk flavor.

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APPENDIX A: Supplementary Tables

Supplementary Table S1. Fatty acid composition of TMR of experimental diets and field peas

Item	Control	LP[1]	HP[2]	Field Peas
C12:0	0.24	0.25	0.26	0.07
C14:0	0.89	0.90	0.93	0.28
C15:0	0.19	0.19	0.18	0.21
C16:0	43.8	45.3	49.0	13.5
C16:1	0.70	0.70	0.71	0.11
C17:0	0.30	0.30	0.28	0.25
C18:0	4.57	4.43	4.08	3.87
C18:1 *cis-9*	18.3	17.8	16.9	23.45
C18:1 *cis-11*	1.00	0.98	0.92	0.59
C18:2 n6	22:0	21.3	19.5	50.1
C18:3 n6	0.08	0.07	0.12	0.07
C18:3 n3	7.46	7.27	5.67	7.04
C20:0	0.48	0.63	0.48	0.45

[1] LP = 3.9 % DM peas

[2] HP = 7.8 % DM peas

Supplementary Table S2. Proportions of saturated and monounsaturated long chain fatty acids (MUFA; g/100 g of FA) of lactating dairy cows fed experimental diets

	Treatment				Significance of contrasts	
Item	**Control**	**LP[1]**	**HP[2]**	**SEM[3]**	**Linear** ***P-values***	**Quadratic** ***P-values***
C18:0	8.91	8.13	8.39	0.34	0.24	0.17
C20:0	0.16	0.13	0.14	0.009	0.30	0.19
C22:0	0.05	0.04	0.04	0.002	0.68	0.25
C24:0	0.03	0.02	0.04	0.004	0.24	0.24
Total saturated	9.16	8.34	8.63	0.35	0.26	0.16
C18:1 *trans-4* [4]	0.03	0.02	0.02	0.001	0.45	0.57
C18:1 *trans-5* [4]	0.02	0.02	0.01	0.001	0.01	0.50
C18:1 *trans-6-8*	0.24	0.21	0.19	0.007	<0.01	0.28
C18:1 *trans-9*	0.22	0.20	0.18	0.007	<0.01	0.60
C18:1 *trans-10*	0.45	0.43	0.39	0.04	0.09	0.65
C18:1 *trans-11* [4]	0.75	0.62	0.56	0.03	<0.01	0.27
C18:1 *trans-12* [4]	0.35	0.33	0.29	0.01	0.01	0.72
C18:1 *trans-13-14*	0.43	0.43	0.34	0.02	0.06	0.26
C18:1 *trans-15*	0.83	0.88	0.80	0.04	0.82	0.50
C18:1 *trans-16*	0.24	0.20	0.21	0.01	0.05	0.02
C18:1 *cis-6-8*	0.12	0.10	0.12	0.006	0.71	0.17
C18:1 *cis-9-10*	17.9	16.9	16.3	0.50	0.05	0.75
C18:1 *cis-11*	1.11	1.28	1.09	0.05	0.88	0.07
C18:1 *cis-12*	0.56	0.62	0.52	0.03	0.58	0.20
C18:1 *cis-13*	0.10	0.13	0.13	0.01	0.08	0.60
C18:1 *cis-14*	0.06	0.05	0.05	0.002	0.39	0.53
C18:1 *cis-15*	0.09	0.09	0.09	0.008	0.45	0.47
C20:1 *cis-9*	0.12	0.10	0.11	0.004	0.19	0.15
C22:1 *cis-13*	0.02	0.01	0.02	0.001	0.61	0.28
C24:1	0.009	0.008	0.014	0.001	0.23	0.29
Total MUFA	23.7	22.7	21.5	0.65	0.05	0.96

[1] LP = 3.9 % DM peas

[2] HP = 7.8 % DM peas

[3] SEM = standard error mean

[4] Data were $\log_{10}$-transformed prior to statistical analysis, but LSM are presented in their original form

Supplementary Table S3. Proportions of polyunsaturated long chain fatty acids (PUFA; g/100 g of FA) of lactating dairy cows fed experimental diets

	Treatment				Significance of contrasts	
Item	**Control**	**LP[1]**	**HP[2]**	**SEM[3]**	**Linear** ***P-values***	**Quadratic** ***P-values***
C18:2 *trans-9, trans-12*	0.01	0.01	0.01	0.0004	0.57	0.11
C18:2 *cis-12, trans-8*	0.29	0.27	0.25	0.009	0.02	0.83
C18:2 c*is-13, trans-8*	0.08	0.07	0.07	0.003	0.02	0.97
C18:2 *cis-9, trans-12*	0.06	0.06	0.05	0.002	0.02	0.90
C18:2 *cis-12, trans-9*	0.03	0.03	0.02	0.001	0.77	0.54
C18:2 *cis-15, trans-11*	0.04	0.04	0.04	0.002	0.01	0.61
C18:2 *cis-9, trans-11* (CLA)	0.41	0.35	0.31	0.01	<0.01	0.47
C18:2 *cis-12, trans-10*	0.009	0.009	0.009	0.0005	0.46	0.72
C18:3n6	0.04	0.03	0.03	0.001	0.92	0.91
C18:3n3	0.49	0.44	0.42	0.01	0.02	0.59
C18:3 *cis-9, cis-15, trans-11*	0.01	0.01	0.01	0.001	0.18	0.59
C18:4n3	0.01	0.01	0.01	0.0006	0.55	0.18
C20:2n6	0.05	0.04	0.04	0.002	0.18	0.20
C20:3n6	0.11	0.10	0.11	0.005	0.94	0.31
C20:3n3	0.006	0.006	0.007	0.0003	0.30	0.25
C20:4n6	0.14	0.13	0.13	0.006	0.60	0.52
C20:4n3	0.01	0.01	0.01	0.001	0.54	0.56
C20:5n3	0.04	0.03	0.04	0.001	0.40	0.08
C22:2n6	0.007	0.006	0.008	0.0003	0.23	0.24
C22:3n3 [4]	0.006	0.006	0.008	0.0004	0.01	0.01
C22:4n6	0.02	0.02	0.02	0.001	0.24	0.24
C22:5n3	0.06	0.05	0.05	0.003	0.36	0.24
C22:6n3	0.009	0.009	0.011	0.0006	0.10	0.22
Total PUFA	2.02	1.81	1.75	0.05	0.02	0.43

[1] LP = 3.9 % DM peas

[2] HP = 7.8 % DM peas

[3] SEM = standard error mean

[4] Data were $\log_{10}$-transformed prior to statistical analysis, but LSM are presented in their original form

Supplementary Table S4. Effects of increasing rate of pea inclusion in lactating dairy cow diets on dry matter intake (DMI), rumination time, and blood serum metabolite concentrations

	Treatment				
Item	**Control**	**LP[1]**	**HP[2]**	**SEM[3]**	**Significance, *P-values***
DMI, kg/day	23.7[b]	24.8[a]	24.3[a]	0.58	0.03
Rumination time, min/d	558	561	547	9.48	0.66
Urea, mmol/L	4.20[b]	4.28[ab]	4.46[a]	0.08	0.04
Glucose, mmol/L	3.64	3.65	3.67	0.03	0.75
BHBA[4], mmol/L	0.80	0.85	0.85	0.02	0.28
NEFA[5,6], mmol/L	0.10	0.10	0.08	0.007	0.20

[1] LP = 3.9 % DM peas

[2] HP = 7.8 % DM peas

[3] SEM = standard error mean

[4] BHBA = beta-hydroxybutyrate

[5] NEFA = non-esterified fatty acids

[6] Data were $\log_{10}$-transformed prior to statistical analysis, but LSM are presented in their original form

[a, b] LSM with different letters within rows differ using the Tukey test ($p < 0.05$)

Supplementary Table S5. Effects of increasing rate of pea inclusion in lactating dairy cow diets on apparent total tract digestibility coefficients (ADC) of dry matter (DM), crude protein (CP) and neutral detergent fiber (NDF)

	Treatment				
ADC	**Control**	**LP[1]**	**HP[2]**	**SEM[3]**	**Significance, *P-values***
DM, %	71.4[a]	69.4[a]	64.7[b]	0.007	0.03
CP, %	69.7[a]	67.7[b]	61.8[c]	0.008	0.02
NDF, %	58.2[a]	56.1[a]	47.8[b]	0.01	<0.01

[1] LP = 3.9 % DM peas

[2] HP = 7.8 % DM peas

[3] SEM = standard error of mean.

[a, b, c] means with different superscripts within rows differ using the Tukey test ($p < 0.05$)

Supplementary Table S6. Effects of increasing rate of pea inclusion in lactating dairy cow diets on milk yield, composition, and feed efficiency

	Treatment				
Item	**Control**	**LP[1]**	**HP[2]**	**SEM[3]**	**Significance, *P-values***
Milk yield[4], kg/d	35.8	36.6	35.8	1.18	0.68
Fat, %	4.16	4.22	4.38	0.10	0.13
Fat, kg/d	1.49	1.53	1.56	0.06	0.31
Protein, %	3.40	3.40	3.38	0.04	0.52
Protein, kg/d	1.21	1.24	1.20	0.04	0.55
Lactose, %	4.62	4.60	4.63	0.02	0.34
MUN[5], mg/dl	11.4[b]	11.2[b]	12.7[a]	0.39	<0.01
ECM[6], kg/d	39.8	40.7	40.5	1.35	0.62
Feed efficiency	1.68	1.64	1.65	0.02	0.59

[1] LP = 3.9 % DM peas

[2] HP = 7.8 % DM peas

[3] SEM = standard error of mean

[4] Data were $\log_{10}$-transformed prior to statistical analysis, but LSM are presented in their original form

[5] MUN = milk urea nitrogen

[6] ECM = energy corrected milk

[a, b] LSM with different superscripts within rows differ using the Tukey test ($p < 0.05$)

Supplementary Table S7. Effects of increasing rate of pea inclusion in lactating dairy cow diets on proportions of *de novo* and C16 milk fatty acids (g/100 g of FA)

	Treatment				
Item	**Control**	**LP[1]**	**HP[2]**	**SEM[3]**	**Significance, *P-values***
C6:0	1.92	1.92	1.95	0.05	0.95
C8:0	1.12	1.10	1.12	0.03	0.90
C10:0	2.45	2.40	2.47	0.07	0.81
C10:1	0.26	0.26	0.25	0.008	0.91
C11:0	0.06	0.07	0.07	0.005	0.45
C12:0	2.98	2.93	3.00	0.09	0.88
C12:1	0.08	0.08	0.08	0.003	0.85
C14:0	10.3	10.3	10.3	0.29	0.99
C14:1 *cis-9*[4]	1.01	1.01	0.98	0.04	0.81
C14:1 *cis-11*	0.06	0.05	0.05	0.003	0.56
C16:0	34.7[b]	37.1[a]	37.9[a]	0.52	<0.01
C16:1 *trans-9*[4]	0.06	0.05	0.05	0.002	0.22
C16:1 *cis-9*	1.54[y]	1.65[x]	1.60[xy]	0.04	0.08
C16:1 *cis-11*	0.05	0.05	0.04	0.002	0.38
C16:1 *cis-13*	0.14	0.15	0.14	0.005	0.71
Total C16	36.7[b]	39.0[a]	39.8[a]	0.53	<0.01

[1] LP = 3.9 % DM peas

[2] HP = 7.8 % DM peas

[3] SEM = standard error of mean

[4] Data were $\log_{10}$-transformed prior to statistical analysis, but LSM are presented in their original form

[a, b] LSM with different superscripts within rows differ using the Tukey test ($p < 0.05$)

[x, y] LSM with different alphabets within rows tend to differ using the Tukey test ($p \leq 0.10$)

Supplementary Table S8. Proportions of saturated and monounsaturated long chain fatty acids (MUFA; g/100 g of FA) of lactating dairy cows fed experimental diets

	Treatment				
Item	**Control**	**LP[1]**	**HP[2]**	**SEM[3]**	**Significance, *P-values***
C18:0	8.91	8.13	8.39	0.34	0.20
C20:0	0.16	0.13	0.14	0.009	0.25
C22:0	0.05	0.04	0.04	0.002	0.47
C24:0	0.03	0.02	0.04	0.004	0.27
Total saturated	9.16	8.34	8.63	0.35	0.19
C18:1 *trans-4* [4]	0.03	0.02	0.02	0.001	0.62
C18:1 *trans-5* [4]	0.02[a]	0.02[ab]	0.01[b]	0.001	0.03
C18:1 *trans-6-8*	0.24[a]	0.21[b]	0.19[b]	0.007	<0.01
C18:1 *trans-9*	0.22[a]	0.20[b]	0.18[b]	0.007	0.02
C18:1 *trans-10*	0.45	0.43	0.39	0.04	0.22
C18:1 *trans-11* [4]	0.75[a]	0.62[b]	0.56[b]	0.03	<0.01
C18:1 *trans-12* [4]	0.35[a]	0.33[a]	0.29[b]	0.01	0.04
C18:1 *trans-13-14*	0.43	0.43	0.34	0.02	0.10
C18:1 *trans-15*	0.83	0.88	0.80	0.04	0.77
C18:1 *trans-16*	0.24[a]	0.20[b]	0.21[ab]	0.01	0.01
C18:1 *cis-6-8*	0.12	0.10	0.12	0.006	0.37
C18:1 *cis-9-10*	17.9	16.9	16.3	0.50	0.13
C18:1 *cis-11*	1.11	1.28	1.09	0.05	0.18
C18:1 *cis-12*	0.56	0.62	0.52	0.03	0.39
C18:1 *cis-13*	0.10	0.13	0.13	0.01	0.18
C18:1 *cis-14*	0.06	0.05	0.05	0.002	0.55
C18:1 *cis-15*	0.09	0.09	0.09	0.008	0.54
C20:1 *cis-9*	0.12	0.10	0.11	0.004	0.15
C22:1 *cis-13*	0.02	0.01	0.02	0.001	0.47
C24:1	0.009	0.008	0.014	0.001	0.29
Total MUFA	23.7	22.7	21.5	0.65	0.16

[1] LP = 3.9 % DM peas

[2] HP = 7.8 % DM peas

[3] SEM = standard error of mean

[4] Data were $\log_{10}$-transformed prior to statistical analysis, but LSM are presented in their original form

[a, b] LSM with different alphabets within rows differ using the Tukey test ($p < 0.05$)

Supplementary Table S9. Proportions of polyunsaturated long chain fatty acids (PUFA; g/100 g of FA) of lactating dairy cows fed experimental diets

	DIET				
Item	**Control**	**LP[1]**	**HP[2]**	**SEM[3]**	**Significance, *P-values***
C18:2 *trans-9, trans-12*	0.01	0.01	0.01	0.0004	0.23
C18:2 *cis-12, trans-8*	0.29[a]	0.27[ab]	0.25[b]	0.009	0.07
C18:2 c*is-13, trans-8*	0.08 [x]	0.07[xy]	0.07[y]	0.003	0.07
C18:2 *cis-9, trans-12*	0.06[x]	0.06[xy]	0.05 [y]	0.002	0.06
C18:2 *cis-12, trans-9*	0.03	0.03	0.02	0.001	0.80
C18:2 *cis-15, trans-11*	0.04[a]	0.04[ab]	0.04[b]	0.002	0.04
C18:2 *cis-9, trans-11* CLA	0.41[a]	0.35[b]	0.31[b]	0.01	<0.01
C18:2 *cis-12, trans-10*	0.009	0.009	0.009	0.0005	0.70
C18:3n6	0.04	0.03	0.03	0.001	0.98
C18:3n3	0.49 [x]	0.44[xy]	0.42 [y]	0.01	0.06
C18:3 *cis-9, cis-15, trans-11*	0.01	0.01	0.01	0.001	0.34
C18:4n3	0.01	0.01	0.01	0.0006	0.33
C20:2n6	0.05	0.04	0.04	0.002	0.17
C20:3n6	0.11	0.10	0.11	0.005	0.58
C20:3n3	0.006	0.006	0.007	0.0003	0.32
C20:4n6	0.14	0.13	0.13	0.006	0.70
C20:4n3	0.01	0.01	0.01	0.001	0.62
C20:5n3	0.04	0.03	0.04	0.001	0.15
C22:2n6	0.007	0.006	0.008	0.0003	0.26
C22:3n3 [4]	0.006[b]	0.006[b]	0.008[a]	0.0004	<0.01
C22:4n6	0.02	0.02	0.02	0.001	0.24
C22:5n3	0.06	0.05	0.05	0.003	0.15
C22:6n3	0.009	0.009	0.011	0.0006	0.14
Total PUFA	2.02 [a]	1.81[ab]	1.75 [b]	0.05	0.05

[1] LP = 3.9 % DM peas
[2] HP = 7.8 % DM peas
[3] SEM = standard error of mean
[4] Data were $\log_{10}$-transformed prior to statistical analysis, but LSM are presented in their original form
[a, b] LSM with different alphabets within rows differ using the Tukey test ($p < 0.05$)
[x, y] LSM with different alphabets within rows tend to differ using the Tukey test ($p \leq 0.10$)

Supplementary Table S10. Effects of increasing rate of pea inclusion in lactating dairy cow diets on ruminal pH, ammonia nitrogen (NH_3-N) and volatile fatty acid (VFA) concentrations

	Treatment				
Item	**Control**	**LP[1]**	**HP[2]**	**SEM[3]**	**Significance, *P-values***
pH	6.03	6.10	5.97	0.03	0.24
Acetate, mmol/L	56.4	56.6	57.0	0.82	0.95
Propionate[4], mmol/L	17.3	17.6	18.1	0.41	0.78
Butyrate, mmol/L	12.4	12.9	12.8	0.31	0.66
Valerate, mmol/L	1.39	1.46	1.57	0.04	0.14
BCVFA[5], mmol/L	1.26	1.37	1.42	0.03	0.12
Total VFA[4], mmol/L	88.7	89.9	90.9	1.35	0.87
Ac/Pr[6]	3.27	3.24	3.20	0.05	0.74
NH_3-N, mg/dL	6.17^{y}	6.50^{xy}	8.67^{x}	0.53	0.05

[1] LP = 3.9 % DM peas

[2] HP = 7.8 % DM peas

[3] SEM = standard error mean

[4] Data were log_{10}-transformed prior to statistical analysis, but LSM are presented in their original form

[5] Branched chain VFA = isobutyric acid + isovaleric acid

[6] Ac/Pr = acetate to propionate ratio

[x, y] LSM with different superscripts within rows tend to differ using the Tukey test ($p \leq 0.10$)

Supplementary Table S11. Differential abundance analysis of the microbiota data at the family level in lactating dairy cows fed experimental diets (3.9 % DM peas, LP; and 7.8 % DM peas, HP)

Feature	Treatment	Coef	Stderr	p-values	q-values
X.Eubacterium..coprostanoligenes.group	HP	-0.33	0.191	0.09	0.76
Anaerovoracaceae	HP	-0.13	0.072	0.09	0.76
Bifidobacteriaceae	LP	-1.42	0.755	0.08	0.76
Eubacteriaceae	LP	-1.04	0.531	0.09	0.76
F082	HP	0.281	0.129	0.04	0.76
Succinivibrionaceae	LP	-1.96	1.062	0.09	0.76
Ruminococcaceae	LP	-0.18	0.113	0.13	0.86
Fibrobacteraceae	HP	-1.04	0.742	0.18	0.87
Rikenellaceae	HP	0.081	0.057	0.17	0.87
Selenomonadaceae	LP	-0.31	0.222	0.17	0.87
Anaerovoracaceae	LP	-0.08	0.068	0.25	0.92
Lachnospiraceae	HP	-0.03	0.023	0.22	0.92
Prevotellaceae	HP	-0.04	0.031	0.23	0.92
X.Eubacterium..coprostanoligenes.group	LP	-0.06	0.181	0.75	0.94
Acholeplasmataceae	LP	-1.48	2.177	0.57	0.94
Acholeplasmataceae	HP	-1.3	1.778	0.54	0.94
Acidaminococcaceae	LP	-0.26	0.234	0.28	0.94
Acidaminococcaceae	HP	0.028	0.247	0.91	0.94
Anaerolineaceae	HP	-0.09	0.729	0.9	0.94
Atopobiaceae	LP	0.243	0.654	0.71	0.94
Atopobiaceae	HP	-0.39	0.679	0.57	0.94
Bacteroidales.RF16.group	LP	0.012	0.112	0.92	0.94

Bacteroidales.RF16.group	HP	0.046	0.119	0.7	0.94
Bifidobacteriaceae	HP	0.545	0.867	0.54	0.94
Christensenellaceae	LP	-0.01	0.034	0.8	0.94
Christensenellaceae	HP	0.008	0.036	0.83	0.94
Erysipelotrichaceae	LP	-1.97	1.965	0.42	0.94
Erysipelotrichaceae	HP	-2.77	2.406	0.37	0.94
Eubacteriaceae	HP	-0.19	0.65	0.78	0.94
F082	LP	0.039	0.122	0.75	0.94
Fibrobacteraceae	LP	-0.38	0.728	0.61	0.94
Lachnospiraceae	LP	-0.01	0.022	0.73	0.94
Muribaculaceae	LP	-0.07	0.442	0.88	0.94
Muribaculaceae	HP	0.367	0.527	0.49	0.94
Oscillospiraceae	LP	-0.05	0.057	0.43	0.94
Oscillospiraceae	HP	0.013	0.06	0.83	0.94
p.251.o5	LP	-0.12	0.491	0.82	0.94
p.251.o5	HP	0.076	0.491	0.88	0.94
Pirellulaceae	LP	-0.35	0.619	0.6	0.94
Pirellulaceae	HP	0.455	0.906	0.64	0.94
Prevotellaceae	LP	-0.02	0.029	0.5	0.94
Rikenellaceae	LP	-0.04	0.054	0.5	0.94
Ruminococcaceae	HP	0.012	0.119	0.92	0.94
Selenomonadaceae	HP	0.195	0.235	0.41	0.94
Spirochaetaceae	LP	0.437	0.61	0.48	0.94
Spirochaetaceae	HP	0.305	0.652	0.64	0.94
Succinivibrionaceae	HP	1.037	1.12	0.38	0.94

Anaerolineaceae	LP	-0.05	0.729	0.95	0.95

Supplementary Table S12. Differential abundance analysis of the microbiota data at the genus level in lactating dairy cows fed experimental diets (3.9 % DM peas, LP; and 7.8 % DM peas, HP)

Feature	Treatment	Coef	Stderr	p-value	q-value
X.Clostridium..methylpentosum.group	LP	-0.126	0.755	0.87	0.99
X.Clostridium..methylpentosum.group	HP	0.059	0.816	0.94	0.99
X.Eubacterium..brachy.group	LP	0.721	0.601	0.3	0.99
X.Eubacterium..brachy.group	HP	-1.256	0.601	0.11	0.99
X.Eubacterium..cellulosolvens.group	LP	-0.547	1.096	0.63	0.99
X.Eubacterium..cellulosolvens.group	HP	0.344	1.096	0.76	0.99
X.Eubacterium..ruminantium.group	LP	-1.939	2.404	0.47	0.99
X.Eubacterium..ruminantium.group	HP	0.263	2.404	0.92	0.99
X.Ruminococcus..gauvreauii.group	LP	0.091	0.088	0.31	0.99
X.Ruminococcus..gauvreauii.group	HP	0.074	0.093	0.43	0.99
Acetitomaculum	LP	0.018	0.04	0.65	0.99
Acetitomaculum	HP	-0.011	0.042	0.79	0.99
Aminicella	LP	-0.409	1.797	0.83	0.99
Anaerobutyricum	LP	0.094	0.088	0.29	0.99
Anaerobutyricum	HP	0.015	0.093	0.87	0.99
Anaeroplasma	LP	-0.644	1.103	0.57	0.99
Anaeroplasma	HP	0.534	1.33	0.7	0.99
Anaerovibrio	LP	1.009	2.428	0.7	0.99
Anaerovibrio	HP	1.911	2.428	0.48	0.99
Atopobium	LP	-2.563	1.019	0.13	0.99
Bacillus	LP	1.768	1.682	0.35	0.99
Bacillus	HP	0.881	1.843	0.66	0.99

Bifidobacterium	LP	1.686	1.063	0.13	0.99
Bifidobacterium	HP	1.797	1.112	0.12	0.99
Blautia	LP	-0.513	0.773	0.52	0.99
Blautia	HP	0.081	0.856	0.93	0.99
Butyribacter	LP	1.158	2.412	0.66	0.99
Butyribacter	HP	-0.082	1.706	0.96	0.99
Butyrivibrio	LP	0.454	0.43	0.3	0.99
Butyrivibrio	HP	0.223	0.443	0.62	0.99
CAG.352	LP	0.494	0.669	0.47	0.99
CAG.352	HP	-0.094	0.669	0.89	0.99
Catenisphaera	LP	2.075	2.278	0.46	0.99
Catenisphaera	HP	-0.663	1.86	0.76	0.99
Christensenellaceae.R.7.group	LP	0.023	0.034	0.49	0.99
Christensenellaceae.R.7.group	HP	0.031	0.036	0.39	0.99
Colidextribacter	LP	0.328	0.389	0.41	0.99
Colidextribacter	HP	0.027	0.399	0.95	0.99
Coprococcus	LP	-0.569	0.598	0.36	0.99
Coprococcus	HP	0.071	0.952	0.94	0.99
Defluviitaleaceae.UCG.011	LP	-3.128	2.111	0.38	0.99
Erysipelotrichaceae.UCG.009	LP	-1.51	1.547	0.43	0.99
Family.XIII.AD3011.group	LP	0.224	0.655	0.74	0.99
Family.XIII.AD3011.group	HP	0.105	0.693	0.88	0.99
Family.XIII.UCG.001	LP	-0.453	1.137	0.76	0.99
Family.XIII.UCG.001	HP	1.917	1.137	0.34	0.99
Fibrobacter	LP	0.886	0.577	0.14	0.99

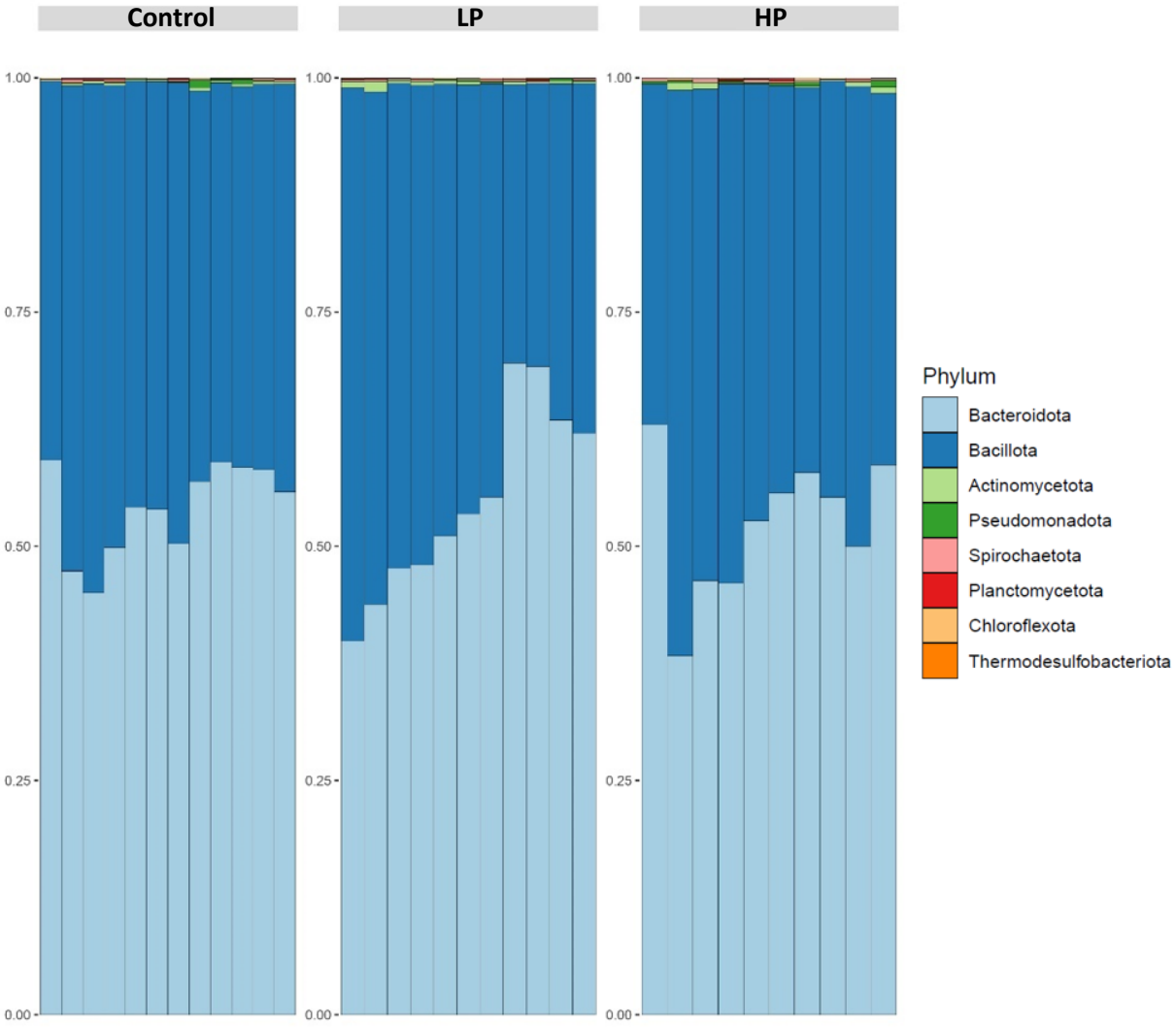
Fibrobacter	HP	1.174	0.626	0.07	0.99
Flexilinea	LP	-0.12	0.634	0.85	0.99
Flexilinea	HP	0.025	0.634	0.97	0.99
Howardella	LP	0.285	0.546	0.6	0.99
Howardella	HP	-0.101	0.619	0.87	0.99
Lachnospira	LP	-1.069	1.394	0.46	0.99
Lachnospira	HP	0.153	1.102	0.89	0.99
Lachnospiraceae.AC2044.group	LP	-0.038	0.336	0.91	0.99
Lachnospiraceae.AC2044.group	HP	-0.227	0.345	0.52	0.99
Lachnospiraceae.ND3007.group	LP	0.016	0.496	0.97	0.99
Lachnospiraceae.ND3007.group	HP	0.186	0.496	0.71	0.99
Lachnospiraceae.NK3A20.group	LP	0.010	0.035	0.76	0.99
Lachnospiraceae.NK3A20.group	HP	-0.016	0.037	0.67	0.99
Lachnospiraceae.UCG.002	LP	0.124	1.013	0.9	0.99
Lachnospiraceae.UCG.002	HP	-0.909	0.966	0.36	0.99
Lachnospiraceae.XPB1014.group	LP	-0.232	0.475	0.63	0.99
Lachnospiraceae.XPB1014.group	HP	-0.273	0.489	0.58	0.99
Lacrimispora	LP	0.088	0.139	0.53	0.99
Lacrimispora	HP	-0.21	0.147	0.16	0.99
Marvinbryantia	LP	-0.049	0.187	0.79	0.99
Marvinbryantia	HP	-0.366	0.197	0.07	0.99
Mogibacterium	LP	-0.022	0.082	0.79	0.99
Mogibacterium	HP	-0.121	0.087	0.17	0.99
Monoglobus	LP	-1.511	1.149	0.26	0.99
Monoglobus	HP	-0.236	1.149	0.85	0.99

Moryella	LP	0.065	0.167	0.7	0.99
NK4A214.group	LP	-0.015	0.057	0.8	0.99
NK4A214.group	HP	0.026	0.061	0.66	0.99
Olsenella	LP	0.070	0.554	0.9	0.99
Olsenella	HP	0.091	0.594	0.88	0.99
Oribacterium	LP	0.234	0.264	0.38	0.99
Oribacterium	HP	-0.033	0.288	0.91	0.99
p.1088.a5.gut.group	LP	0.604	0.71	0.4	0.99
p.1088.a5.gut.group	HP	0.150	0.71	0.83	0.99
Prevotella	LP	0.837	0.858	0.36	0.99
Prevotella	HP	0.714	0.983	0.49	0.99
Prevotellaceae.Ga6A1.group	LP	0.389	0.478	0.42	0.99
Prevotellaceae.Ga6A1.group	HP	-0.122	0.493	0.81	0.99
Prevotellaceae.NK3B31.group	LP	0.138	0.474	0.77	0.99
Prevotellaceae.NK3B31.group	HP	-0.884	0.505	0.09	0.99
Prevotellaceae.UCG.001	LP	-0.015	0.058	0.8	0.99
Prevotellaceae.UCG.001	HP	-0.037	0.061	0.55	0.99
Prevotellaceae.UCG.003	LP	-0.055	0.063	0.39	0.99
Prevotellaceae.UCG.003	HP	-0.027	0.067	0.69	0.99
Prevotellaceae.UCG.004	LP	-0.284	0.503	0.58	0.99
Prevotellaceae.UCG.004	HP	-0.57	0.584	0.34	0.99
Prevotellaceae.YAB2003.group	LP	1.188	0.908	0.21	0.99
Prevotellaceae.YAB2003.group	HP	1.794	0.972	0.09	0.99
probable.genus.10	LP	0.114	0.46	0.81	0.99
probable.genus.10	HP	0.272	0.505	0.59	0.99

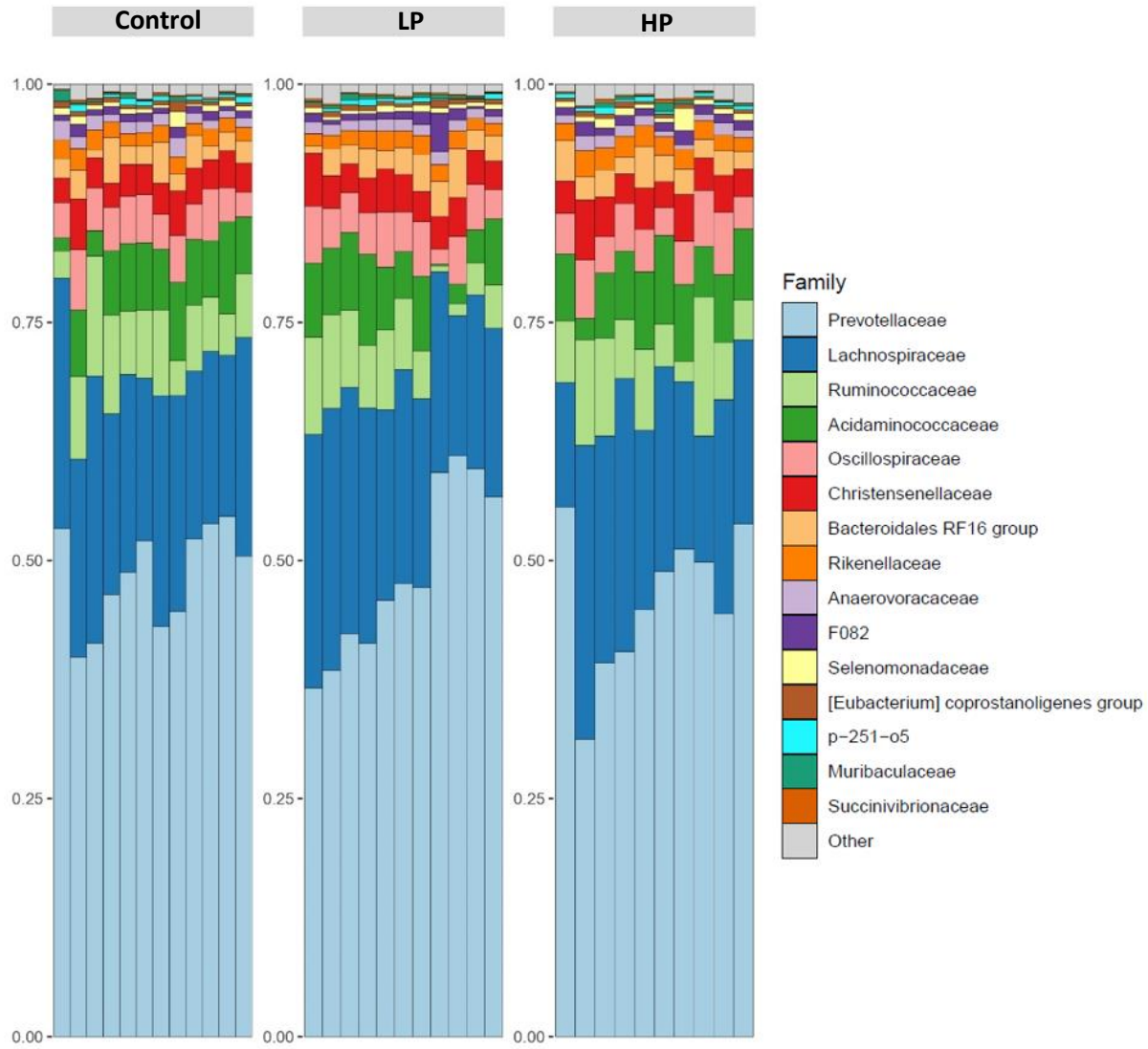
Pseudobutyrivibrio	LP	-0.284	0.512	0.58	0.99
Pseudobutyrivibrio	HP	-0.645	0.512	0.22	0.99
Pseudoramibacter	LP	0.062	0.756	0.93	0.99
Pseudoramibacter	HP	-0.043	0.721	0.95	0.99
Pseudoscardovia	LP	-1.166	0.463	0.05	0.99
Pseudoscardovia	HP	0.168	0.535	0.77	0.99
Rikenellaceae.RC9.gut.group	LP	0.011	0.05	0.82	0.99
Rikenellaceae.RC9.gut.group	HP	0.094	0.053	0.09	0.99
Ruminococcus	LP	-0.174	0.126	0.18	0.99
Ruminococcus	HP	0.035	0.133	0.79	0.99
Saccharofermentans	LP	-0.008	0.247	0.98	0.99
Schwartzia	LP	0.322	0.234	0.18	0.99
Schwartzia	HP	0.415	0.241	0.1	0.99
Segatella	LP	-0.136	0.285	0.64	0.99
Segatella	HP	-0.434	0.304	0.16	0.99
Selenomonas	LP	-1.216	0.528	0.03	0.99
Selenomonas	HP	0.145	0.544	0.79	0.99
Shuttleworthia	LP	0.242	0.615	0.7	0.99
Shuttleworthia	HP	1.515	0.615	0.04	0.99
Succiniclasticum	LP	-0.142	0.161	0.39	0.99
Succiniclasticum	HP	0.045	0.171	0.79	0.99
Succinivibrionaceae.UCG.001	LP	0.423	1.41	0.77	0.99
Succinivibrionaceae.UCG.001	HP	1.188	1.41	0.42	0.99
Succinivibrionaceae.UCG.002	HP	-0.135	0.684	0.88	0.99
Sutterella	LP	0.154	1.887	0.94	0.99

Sutterella	HP	1.447	1.887	0.52	0.99
Syntrophococcus	LP	0.029	0.453	0.95	0.99
Syntrophococcus	HP	-0.57	0.486	0.25	0.99
Treponema	LP	-0.08	0.364	0.82	0.99
Treponema	HP	-0.43	0.382	0.27	0.99
UCG.001	LP	-0.12	0.222	0.6	0.99
UCG.001	HP	-0.3	0.234	0.21	0.99
UCG.002	HP	-0.9	1.423	0.57	0.99
UCG.004	LP	3.387	0.86	0.16	0.99
UCG.004	HP	3.526	0.86	0.15	0.99
Veillonellaceae.UCG.001	LP	0.541	0.725	0.47	0.99
Veillonellaceae.UCG.001	HP	0.737	0.725	0.33	0.99
Xylanibacter.Prevotella	LP	0.006	0.027	0.83	0.99
Xylanibacter.Prevotella	HP	-0.02	0.029	0.58	0.99
Moryella	HP	-0	0.177	0.99	0.99
Saccharofermentans	HP	0.001	0.261	1	1

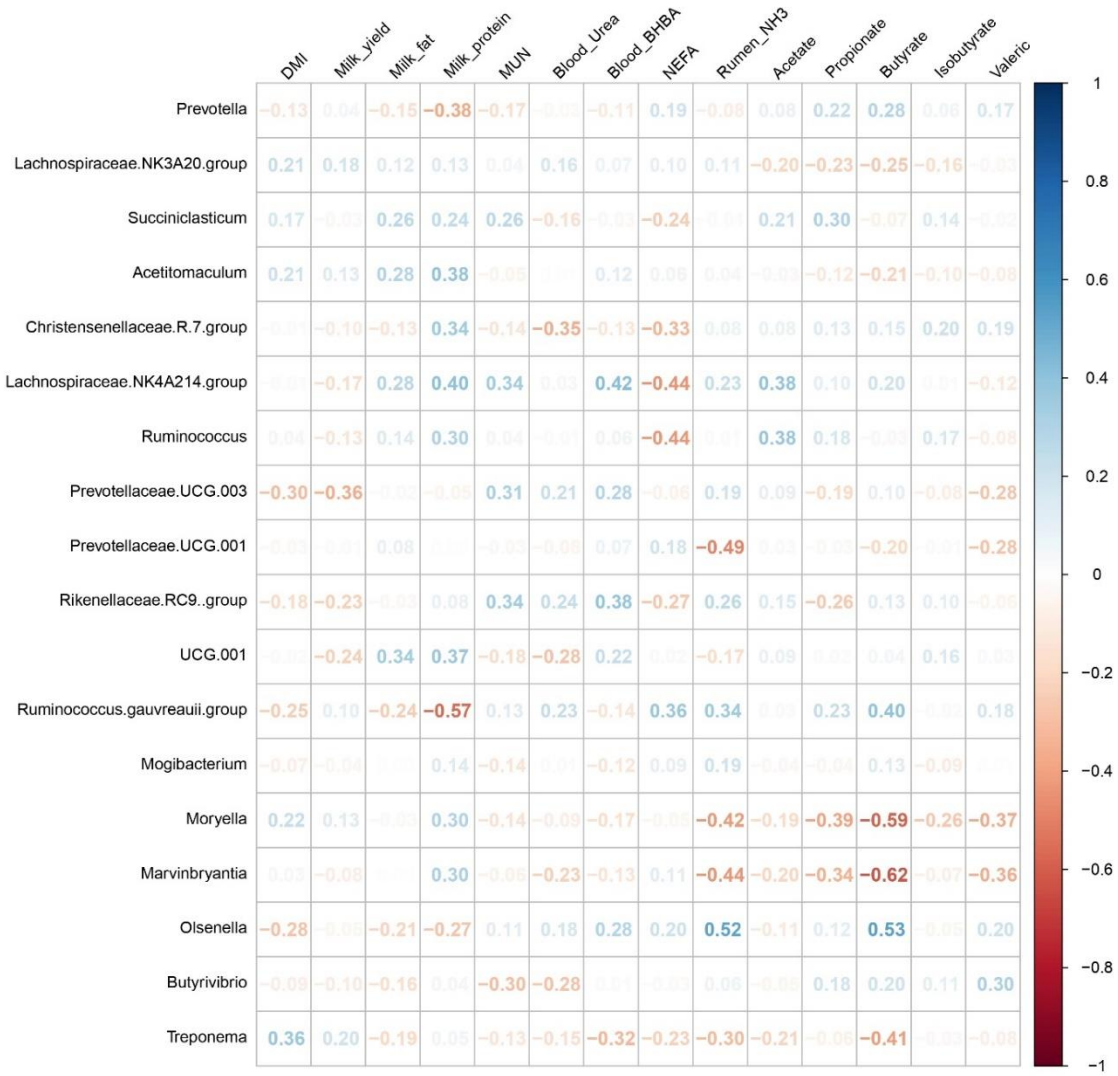
APPENDIX B: Supplementary Figures



Supplementary Figure S1. Relative abundances of ruminal bacteria at the phylum level across rumen samples in lactating dairy cows. HP = 7.8 % DM peas group; LP = 3.9 % DM peas group.



Supplementary Figure S2. Relative abundances of ruminal bacteria at the family level across rumen samples in lactating dairy cows. HP = 7.8 % DM peas group; LP = 3.9 % DM peas group.



Supplementary Figure S3. Spearman's rank correlation coefficient of dairy production and rumen fermentation parameters with rumen bacteria genera in lactating dairy cows. Blue color shows positive correlations, and red color shows negative correlations. Darker colors indicate higher coefficients.