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IMPROVING THE UNDERSTANDING OF DIFFERENT DIETS ON THE CONCENTRATION AND METABOLISM OF THE MAMMALIAN LIGNAN ENTEROLACTONE IN DAIRY CATTLE

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BY

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LIST OF ABBREVIATIONS

This dissertation was written following the Instructions for authors from Journal of Dairy Science (2017). A list of abbreviations and standard units that does not require definition from the instructions for authors was used hereon per University of New Hampshire thesis manual policy. The instructions from Journal of Dairy Science can be found in: http://www.journalofdairyscience.org/pb/assets/raw/Health%20Advance/journals/jods/2016_SF.pdf

The following are a list of abbreviations not defined in the Journal of Dairy Science instructions for authors and that are defined at the first time they appear in each chapter:

EL: enterolactone; ED: enterodiol; FM: flaxseed meal; FO: flaxseed oil; LM: liquid molasses; GRC: ground corn; TBARS: thiobarbituric acid reactive substances; LAR: lariciresinol; MAT: matairesinol; PINO: pinoresinol; SECO: secoisolariciresinol; SDG: secoisolariciresinol diglucoside; ALA: α-linolenic acid; PUFA: polyunsaturated fatty acids; AUC: area under the curve; MR: milk replacer; ER: estrogen receptor; VFA: volatile fatty acids; SOD: superoxide dismutase; GPx: glutathione peroxidase; -CTRL: diet containing: 8% SBM plus 23% ground corn; SUCR: 15% FXM +10.7% ground corn + 5% sucrose; OIL: 15% FXM + 15.4% ground corn + 3% FO; COMBO: 15% FXM +10.2% ground corn + 5% sucrose + 3% FO; NFC: non-fiber carbohydrates; ADF: Acid detergent fiber; NDF: neutral detergent fiber; TMR: total mixed ration, DM: dry matter; OM: organic matter; PUN: plasma urea nitrogen; MUN: milk urea nitrogen; DIM: days in milk; DMI: dry matter intake; BW: body weight.

ABSTRACT

IMPROVING THE UNDERSTANDING OF DIFFERENT DIETS ON THE CONCENTRATION AND METABOLISM OF THE MAMMALIAN LIGNAN ENTEROLACTONE IN DAIRY CATTLE

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Flaxseed *(Linum usitatissimum)* is the richest source of the plant lignan secoisolariciresinol diglucoside (SDG), which is precursor for the synthesis of the mammalian lignans enterolactone (EL) and enterodiol (ED) by the gastrointestinal microbes in mammals. There is a great deal of interest in promoting increased intakes of lignans in humans' diet due to their potential health benefits, especially in the prevention of cardiovascular diseases, hypercholesterolaemia, breast and prostate cancers, and osteoporosis. Consumption of milk and dairy products enriched in EL could be an excellent strategy to increase the intake of lignans by humans. The first (Chapter II) and second (Chapter III) studies presented in this dissertation aimed to evaluate strategies to improve the concentration of EL in milk of dairy cows.

In Chapter II, we evaluated the effects of replacing ground corn (GRC) with incremental amounts of liquid molasses (LM) on production, milk composition (fat, true protein, lactose, EL, urea N, fatty acids), plasma concentrations of antioxidant enzymes and urea N, and apparent total-tract digestibility of nutrients in Jersey cows fed FM. Sixteen multiparous organically-certified Jersey cows averaging (means ± standard deviation) 99 ± 41 d in milk and 462 ± 38 kg of body weight were randomly assigned to treatment sequences in a replicated 4 × 4 Latin square design with 14 d for diet adaptation and 7 d for data and sample collection. Diets were fed as total mixed rations and consisted (dry matter basis) of 52% grass-legume baleage, 8% grass hay, 8.5% soyhulls, 2.5% roasted soybean, and 15% FM. Ground corn was totally replaced by

increasing amounts of LM at 0, 4, 8, or 12% of the diet dry matter. Orthogonal polynomials were used to test linear, quadratic, and cubic effects in response to LM supplementation using the MIXED procedure of SAS. Milk concentration of EL tended to respond cubically when replacing GRC by incremental amounts of LM in cows fed FM. The plasma activities of the antioxidant enzymes glutathione peroxidase and catalase did not differ, but superoxide dismutase activity tended to respond cubically with feeding increasing amounts of LM at expense of GRC. Dry matter intake and yields of milk and milk fat, true protein, and lactose decreased linearly with substituting GRC for LM. Whereas the concentrations of milk fat and milk true protein did not differ across treatments, milk lactose content decreased linearly. Feeding incremental levels of LM reduced linearly the milk concentration of urea N and the amount of N excreted in urine, and tended to decrease linearly the concentration of plasma urea N. Apparent total-tract digestibilities of dry matter, organic matter, and neutral and acid detergent fiber did not differ across treatments, while digestibility of crude protein decreased linearly. Milk fatty acids profile was substantially changed most notably by linear increases in *cis*-9, *trans*-11 18:2, *cis*-9, *cis*-12, *cis*-15 18:3, Σ odd-chain fatty acids, and the *trans*-11/*trans*-10 ratio, and linear decreases in *cis*-9 18:1 and *cis*-9, *cis*-12 18:2 when replacing GRC for incremental amounts of LM.

In Chapter III was evaluated the effects of feeding flaxseed oil or sucrose alone or in combination on production, milk composition (fat, true protein, lactose, EL, urea N) plasma concentrations of thiobarbituric acid reactive substances (TBARS) and urea N, and apparent total-tract digestibility of nutrients in Holstein cows fed FM. Sixteen multiparous (4 ruminally-cannulated) Holstein cows averaging 94 ± 37.6 d in milk and 680 ± 79.1 kg of BW at the beginning of the study were used. Cows were randomly assigned to treatment sequences in a 4 replicated 4 × 4 Latin square design. Each experimental period lasted 25 d with 18 d for diet

adaptation and 7 d for data and sample collection. Treatments were fed (dry matter basis) as total-mixed rations consisting of a 60:40 forage-to-concentrate ratio and included: a negative control diet (**-CTRL**; 8% SBM plus 23% ground corn); 15% FM +10.7% ground corn + 5% sucrose (**SUCR**); 15% FM + 15.4% ground corn + 3% flaxseed oil (**OIL**); and 15% FM +10.2% ground corn + 5% sucrose + 3% FO (**COMBO**). It was observed that cows fed FM had lower dry matter intake (DMI) compared with that fed soybean meal. Within cows fed FM, the reduction in DMI was greatest in cows fed the OIL diet with no difference between SUCR and COMBO treatments. Milk yield did not differ between cows fed the -CTRL diet and those fed the SUCR and OIL diets. However, a negative associative effect was observed for milk production when FM was supplemented with sucrose and FO. The concentration and yield of milk fat decreased when FO was added to FM. No effects of treatments were observed regarding concentrations and yield of milk true protein, and concentration of milk lactose. However, lactose yield and MUN tended to decrease in the COMBO diet. Digestibility of DM and OM were lower in cows fed FM diets than in those offered the –CRTL treatment. Digestibility of ADF was greatest in –CRTL, intermediate in SUCR, and lowest in OIL and COMBO and no differences across treatments were observed for the apparent total-tract digestibilities of NDF and CP. As expected, the concentration and yield of milk EL were both greater in cows fed FM diets than those fed soybean meal. No difference in milk EL was observed when FM was supplemented with either sucrose or FO alone or their combination (COMBO diet), suggesting no synergistic effects of sucrose and FO in the conversion of SDG to EL in the rumen.

A second aim of this dissertation (Chapter IV) was to determine the pharmacokinetics of EL in newborn dairy calves fed milk replacer (MR) or EL-enriched milk. In newborn calves, suckling stimulates the reflex closure of the esophageal groove so that milk or milk replacer bypass the reticulo-rumen down to the abomasum. Thus, calves may be used as a model to make inferences about the pharmacokinetics of EL in simple-stomach mammals including humans. The objective of this study was to determine the pharmacokinetics of EL from MR or EL-enriched milk consumed by newborn Holstein calves. Twenty Holsteins calves (n = 10 males and 10 females) were used from birth to d 7 of life. The 10 calves born from multiparous cows received 4 L of colostrum using nipple bottles. Whereas, the 10 calves born from primiparous cows were fed 4 L of stored colostrum from multiparous cows when available or colostrum replacer. On d 5 of life, calves were administered 2 L of milk replacer (n = 10; Low-EL treatment: 123 nmol/L EL) or 2 L of EL-enriched milk (n = 10; High-EL treatment: 481 nmol/L EL) during the morning feeding (0700 h). Blood samples were taken from the jugular vein before (0 h) and at 0.5, 1, 1.5, 2, 2.5, 3, 4, 6, 8, 10, 12, 24, and 48 h after oral administration of treatments. The area under the curve for the plasma concentration of EL was analyzed according to the trapezoidal rule between 0 and 12 h after treatment administration, and it was greater in High- (26 nmol/L × h) than Low-EL calves (4.30 nmol/L × h). Similarly, the maximum concentration of EL in plasma was greater in High- (5.06 nmol/L) vs. Low-EL calves (1.95 nmol/L). Furthermore, the time after treatment intake to reach maximum plasma concentration of EL was faster in High- (4.31 h) compared with Low-EL (4.44 h) treatment. Calves were able to absorb EL, thus indicating that EL-enriched milk can be potentially used as source of EL to pre-weaned ruminants.

CHAPTER I: REVIEW OF LITERATURE

Introduction

Flaxseed (*Linum usitatissimum*) is a blue flowering annual herb that belongs to the family Lineaceae (Rubilar et al., 2010; Sigh et al., 2011). The seeds of flaxseed are flat and vary in color from golden yellow to reddish brown (Rubilar et al., 2010). Flaxseed is also referred to as linseed. Usually, the term flaxseed is used to describe flax that is consumed by humans, whereas the term linseed is used to denote flax when it is used for industrial purpose (Rubilar et al., 2010). Flaxseed is cultivated for fiber, medicinal purposes, and as nutritional product in more than 50 countries (Sigh et al., 2011). Currently, Canada is the largest producer in the world and India, China, United States, and Ethiopia can also be cited as important flaxseed growing countries (Sigh et al., 2011; Kajla et al., 2015).

Humans have been consuming flaxseed since ancient times and flaxseed is establishing importance as a functional food mainly due to the presence of three main bioactive compounds: α-linolenic acid (**ALA**), dietary fiber, and lignans (Toure and Xueming, 2010; Kajla et al., 2015).

Flaxseed can be fed to dairy cows as a source of both energy and protein (Petit, 2011). In addition, whole flaxseed and flaxseed oil are excellent sources of ALA and the outer fiber-containing layers of flaxseed is the richest source of lignans, which are polyphenolic compounds known as phytoestrogens (Thompson et al., 1991; Kajla et al., 2015). Supplementation of flaxseed to dairy cows contribute to favorable changes in milk composition for better human health by enhancing nutraceutical compounds. For instance, improved amounts of polyunsaturated fatty acids (**PUFA**) in milk of dairy cows have been reported with feeding extruded flaxseed (Zachut et al., 2010) and flaxseed oil (Caroprese et al., 2010) to dairy cows. Additionally, supplementation with flaxseed oil has been shown to increase the concentration of conjugated linoleic acid in milk of dairy cows (Glasser et al., 2008). Whereas, improved

concentration of mammalian lignans in milk has been reported with feeding flaxseed meal (**FM**), a lignan-rich source, to dairy cows (Petit and Gagnon, 2009; Brito et al., 2015; Lima et al., 2016).

Mammalian lignans are bioactive compounds with a wide range of biological activities, including: antioxidant, antitumor, and weakly estrogen-linked proprieties. There is great deal of interest in promoting the inclusion of lignan-rich foods in human diets due to the potential human health benefits of mamalians lignans, including prevention of cardiovascular diseases, hypercholesterolemia, breast and prostate cancers, menopausal symptoms, and osteoporosis (Murkies et al., 1998; Adlercreutz, 2002).

Secoisolariciresinol diglucoside (**SDG**) is the major lignan in flaxseed, accounting for more than 95% of the total lignans. It is mostly concentrated in the outer fiber-containing layers of the seed (Adlercreutz and Mazur, 1997), thus resulting in greater concentration of SDG in hulls compared to seeds. Secoisolariciresinol diglycoside is precursor for synthesis of the mammalian lignans enterolactone (**EL**) and enterodiol (**ED**) by the gut microbiota in humans (Thompson et al., 1991) and ruminants (Côrtes et al., 2008; Gagnon et al., 2009a). In dairy cows, EL is the predominant mammalian lignan found in the rumen and physiological fluids, including plasma, urine, and milk (Côrtes et al., 2008; Gagnon et al., 2009a).

Saarinen et al. (2002) reported that rats fed pure EL had a 5-fold increase in urinary excretion of EL compared with those fed plant lignans. These findings indicate that prior absorption, plant lignans must be converted to EL by microbes in the colon, whereas deconjugated EL may be passively absorbed along the intestine of mammals (Saarinen et al., 2002). The concentration of EL in milk of dairy cows can be modulated by dietary changes and EL-enriched milk or dairy products could be used as a source of EL for humans (Petit and

Gagnon, 2009; Brito et al., 2015). Humans rely on gut microbes to convert plant lignans to mammalian lignans (Thompson et al., 1991). Therefore, the intake of EL-enriched milk or dairy products may be more efficient in providing EL for humans, than the intake of plant lignans.

Feeding SDG-rich sources to dairy cows is one of the dietary strategies that can be applied to improve milk EL concentration. Indeed, supplementation with FM (Petit and Gagnon, 2009; Brito et al., 2015; Lima et al., 2016) and flaxseed hulls (Gagnon et al., 2009a; Petit et al., 2009) have been reported to improve the concentration of EL in milk of dairy cows. The rumen is the major site for lignans metabolism in cows, where plant lignans are converted to mammalian lignans under the action of ruminal microbes (Cortes et al., 2008; Gagnon et al., 2009). Schogor et al. (2014) investigated lignans metabolism by ruminal microbes in dairy cows and reported that *Prevotella spp.* were the main converters of SDG to secoisolariciresinol (**SECO**), a lignan-derived metabolite that is further metabolized to ED and EL presumably by other ruminal bacteria species. Therefore, changes in the diet that favor the number of *Prevotella spp.* in the rumen could potentially improve the metabolism of plant lignans and contribute to a higher output of EL in milk of dairy cows.

A study conducted previously in our laboratory reported that changes in the carbohydrate profile of FM-based diets fed to dairy cows can potentially be a strategy to alter the output of milk EL. In this study, cows receiving FM and liquid molasses (**LM**), a sucrose-rich source, had higher milk EL concentration than those fed FM and ground corn (**GRC**), a starch-rich source. These findings suggest that LM supplementation may have a greater potential to select ruminal microorganisms with superior capacity to convert plant lignans to mammalian liganans, when compared with GRC (Brito et al., 2015).

Supplementation with flaxseed oil has been reported to improve *Prevotella spp.* abundance in the rumen. A study carried out by Li et al. (2015) showed that when steers were fed with 4% of flaxseed oil the genus *Prevotella* dominated the ruminal bacterial community. Therefore, supplementing flaxseed oil to dairy cows receiving SDG-rich sources, such as FM, may be a dietary strategy to improve EL concentration in milk through a potential improvement on lignans metabolism by favoring the number of *Prevotella spp.* in the rumen.

This dissertation describes two studies that were conducted to evaluate dietary strategies to improve the concentration of EL in milk of dairy cows. The first study, presented on chapter two, evaluated the effects of feeding incremental levels of LM to Jersey cows fed FM-based diets. The second study, presented on chapter three, investigated how feeding flaxseed oil and sucrose alone or their association impacts milk EL concentration of Holstein cows fed FM as the major protein supplement. Finally, the experiment presented on chapter four evaluated the pharmacokinetics of EL in newborn dairy calves fed EL-enriched milk.

This literature review will focus on describing the chemical composition of flaxseed, specifically regarding lignans content while discussing the metabolism of mammalian lignans in both non-ruminant and ruminant animals. Biological activities and potential human health benefits of mammalian lignans will be briefly addressed. Additionally, strategies to improve milk EL concentration and effects of replacing dietary starch with sugars on performance of dairy cows will be discussed as well.

Flaxseed chemical composition

The chemical composition of whole-grain flaxseed is detailed on Table 1. Flaxseed chemical composition varies upon growing environment, genetics, processing conditions, and

method of analysis (Morris, 2007). The major component of flaxseed is its oil. It has around 40% fat found manly as triglycerides (98%) with lower contents of phospholipids (0.9 %) and free fatty acids (0.1%) (Mueller et al., 2010). Flaxseed is a rich source of n-3 fatty acids, especially ALA which can constitute up to 55% of the total fatty acids in flaxseed (Mustafa et al. 2003; Mueller et al., 2010). The content of neutral detergent fibre (**NDF**) of flaxseed is around 30% (Chung et al., 2005). Flaxseed is also a good protein source. It contains around 20% of crude protein (**CP**), mainly globulin (26–58%) and albumin (20–42%). Regarding amino acid profile, flaxseed is rich in arginine, aspartic acid, and glutamic acid, and limiting in lysine (Chung et al., 2005).

Flaxseed can be fed to dairy cows as whole flaxseed, flaxseed oil, and FM. Flaxseed meal is the residue remaining after flaxseed oil extraction. Compared to whole flaxseed, FM is greater in fiber and protein and lower in crude fat (Gagnon et al., 2009). Therefore, FM is fed to dairy cows mainly as a protein source. Chemical composition of FM and other protein supplements fed to dairy cows are detailed in Table 2.

Flaxseed lignans

One of the most interesting characteristics of flaxseed is its content of complex phenols, known as phytoestrogens, primarily lignans. Phytoestrogens are a diverse group of compounds found naturally in many edible plants and their seeds that have a phenolic group shared with estrogenic steroids (Wang et al., 2002). Phytoestrogens are known as plant compounds with estrogen-like biological activity and are classified according to their chemical structure in three major categories: isoflavones, cousmestans, and lignans (Adlercreutz and Mazur, 1997).

Plant lignans are defined as diphenolic compounds produced by the coupling of 2 coniferyl alcohol residues existing in cell wall of high plants (Toureand and Xueming, 2010). They are found in fibrous rich plants: cereals (barley, wheat and oats), vegetables (broccoli, garlic, asparagus and carrots), legumes (bean, lentil and soybean), fruits, berries, tea, and alcoholic beverages (Wang et al., 2002). Flaxseed is the richest source of plant lignans. Flaxseed lignans are mostly concentrated in the outer fiber-containing layers of the seed (Adlercreutz and Mazur, 1997). The concentration of SDG in flaxseed hulls has been reported to be 3.4-times greater than in whole flaxseeds (Cortes et al., 2012). The chemical structure of flaxseed lignans is detailed in Figure 1. Secoisolariciresinol diglycoside is the major lignan of flaxseed, representing over 95% of the total lignans (Daun et al., 2003; Liu et al., 2006). Minor concentrations of other lignans including matairesinol, pinoresinol, lariciresinol, and isolariciresinol are also present in flaxseed (Raffaelli et al., 2002). Table 3 details the concentration of lignans in whole-grain flaxseed. According to Johnsson et al. (2000) SDG concentration in flaxseed ranges from 1.7 to 2.4 % in defatted flour and 0.6 to 1.3 % in whole flaxseed flour. Flaxseed meal SDG concentration were 1.66% and 1.64% of DM in the studies done by Brito et al. (2015) and Petit and Gagnon (2009), respectively.

Lignans metabolism in non-ruminant and ruminant animals

Non-ruminant animals

In mammals, plant lignans are metabolized by the gastrointestinal microbiota to the mammalian lignans: EL and ED (Thompson et al., 1991; Chen et al., 2007; Côrtes et al., 2008; Gagnon et al., 2009) (Figure 2). Figure 3 shows the pathways for mammalian lignans synthesis from different plant lignans. The pathway for conversion of SDG, the major lignan found in

flaxseed, to mammalian lignans is detailed in Figure 4. The conversion of plant SGD to mammalian lignans involves basically 3 steps that take place in the gut of non-ruminant animals: (1) first, SDG is hydrolyzed under the action of intestinal glycosidases to SECO, which is the non-sugar moiety of SGD (Clavel et al., 2006; Chen et al., 2007). *Bacteroides* and *Clostridia* have been reported to release the glucosyl moieties from SDG to yield SECO (Clavel et al. 2006). (2) Further, colonic microbes convert SECO to ED by demethylation and dehydrogenation. (3) Finally, ED can be converted to EL by dihydroxylation (Clavel et al., 2006; Chen et al., 2007). *Peptostreptococcus productus*, *Eubacterium callanderi*, *Eubacterium limosum*, and *Bacteroides methylotrophicum* have been identified as the major bacteria responsible for demethylation reactions, whereas dehydrogenation reactions are carried out mainly by *Eubacterium lentum* (Wang et al., 2000; Clavel et al., 2007). Several *Clostridia* and *Ruminococcus spp*. have been cited as the major microorganisms responsible for converting ED to EL by dihydroxylation as cited above (Clavel et al., 2007).

Mammalian lignans formed from plant lignans by the gut microbiota have three metabolic fates: (1) they are directly excreted in feces, (2) they are taken up by epithelial cells lining of the colon and conjugated with glucuronic acid or sulfate. After conjugation, EL and ED enter the circulation and can eventually be excreted in feces, and (3) they can be absorbed from the gut in their deconjugated form and reach the liver, where free forms are conjugated and released into the bloodstream. Eventually, the conjugated mammalian lignans are excreted into physiological fluids (e.g., plasma and urine) or return to the intestine via enterohepatic circulation (Wang et al., 2000; Landete, 2012). The conjugated forms of mammalian lignans that reach the intestine via enterohepatic circulation are poorly absorbed. The microbial enzyme β-glucuronidase converts mammalians lignans to their deconjugated forms, allowing them to be

reabsorbed in the intestine (Raffaeli et al., 2002). The activity of β-glucuronidase in humans has been attributed to intestinal-dominant bacteria belonging to *Bacteroides, Bifidobacterium, Eubacterium,* and *Ruminococcus* genera (Akao et al., 2000).

Ruminant animals

The metabolism of lignans in ruminant aminals is not completely elucidated. It is known that plant lignans are metabolized to mammalian lignans by both ruminal and fecal microbiota (Cortes et al., 2008). Recent studies have demonstrated that lignans metabolism in ruminants occurs mainly into the rumen, where plant lignans are converted to mammalian lignans by ruminal microbes (Cortes et al., 2008; Gagnon et al., 2009a). The study done by Gagnon et al. (2009a) was the first *in vivo* study to investigate the role of ruminal microorganisms in flaxseed lignans metabolism in lactating dairy cows. Ruminally-cannulated dairy cows were assigned to the following experimental treatments: (1) Flaxseed oil and flaxseed hulls administration into the rumen and water infusion in the abomasum; (2) oil and hulls administration into the abomasum; (3) oil infusion in the abomasum and hulls placed in the rumen; or (4) oil placed in the rumen and hulls administered in the abomasum. Enterolactone concentration on milk and urine were 12 and 16 times greater, respectively, with flaxseed hulls administration in the rumen compared to administration of flaxseed hulls in the abomasum. Similarly, plasma EL concentration was three times higher in cows receiving flaxseed hulls in the rumen than those receiving flaxseed hulls in the abomasum. These results demonstrated that ruminal microbiota plays an important role in converting flaxseed lignans to mammalian lignans in dairy cows.

Despite the importance of ruminal microbes on lignans metabolism in dairy cows, just few studies have investigated lignans metabolism in the rumen and how dietary changes could impact this process. Recently, Schogor et al. (2014) studied lignans metabolism using selected

pure cultures of ruminal bacteria incubated in vitro with SDG. It was reported that 11 ruminal bacteria, mainly *Prevotella spp.* were able to convert SDG to SECO, which is formed as an intermediate in the ruminal metabolim of SDG to EL. These findings suggest that *Prevotella spp*. may play an important role in lignan metabolism in dairy cows (Schogor et al., 2014).

Enterolactone has been identified as the major lignan metabolite present in ruminal fluid, urine, plasma, and milk of dairy cows (Petit and Gagnon, 2009; Gagnon et al., 2009a; Brito et al., 2015). Gagnon et al. (2009b) investigated the length of time to obtain peak EL concentration in milk of dairy cows fed FM (20% of diet DM). The length of time need for EL to return to baseline level was also evaluated in this study. It was reported that the conversion of SDG to EL and the transfer of EL to the mammary gland are established after one week of FM supplementation, whereas milk concentration of EL returned to baseline level after one week of FM deprivation.

Studies with have investigated β- glucuronidase activity in lignans metabolism in dairy cows. Petit et al., (2009) evaluated the effect of feeding monensin and flaxseed hulls on β-glucuronidase activity in rumen fluid and feces. Monensin is known to decrease the growth of Gram positive bacteria and could potentially impact β- glucuronidase activity, considering that strains of ruminal bacteria with β- glucuronidase activity (e.g. Ruminococcus and Eubacterium) are Gram positive bacteria (Jenab & Thompson, 1996). Indeed, the activity of β- glucuronidase in ruminal fluid tended to decrease when cows received monensin. Flaxseed hulls supplementation decreased β- glucuronidase activity in both ruminal fluid and feces (Petit et al.,2009). Similar results were reported with ruminal infusion of flaxseed oil in the study done by Gagnon et al. (2009a) and could be explained by the high content of omega-3 FA o these flax products which can negatively affect the growth of ruminal bacteria (Maia et al. 2007). A

subsequent study, (Lima et al., 2016) investigated the effects of dietary FM and abomasal infusion of flaxseed oil and their interaction on activity of β-glucuronidase. It was reported that abomasal infusion of flaxseed oil, which is a PUFA rich source, had no effect on β-glucuronidase activity. These results suggest that polyunsaturated fatty acids do not interfere with the absorption of mammalian lignans in ruminants. No effect of FM supplementation was reported on activity of β- glucuronidase. The author also reported higher activity of β- glucoronidase in feces than in ruminal fluid suggesting that the deconjugation reactions may be more important in the large intestine than in the rumen of ruminant animals (Lima et al., 2016).

Biological activities and potential human health benefits of mammalian lignans

There is a growing interest in promoting the inclusion of lignans-rich sources in human diets due to the potential health benefits of mammalian lignans. Flaxseed lignans and the mammalian lignans ED and EL are biologically active substances that elicit a wide range of biological activities including weak estrogenic and cardioprotective effects, as well as antiestrogenic, antioxidant, anti-inflammatory, and anticarcinogenic properties (Adolphe et al., 2010; Högger, 2013; Imran et al., 2015; Landete, 2012).

Lignans and mammalian lignans are known to be strong antioxidants and their antioxidant proprieties are presumably the main reason for the anticancer activity of these components in humans (Prasad, 2000; Landete, 2012). Prasad (2000) studied the antioxidant activity of SECO, ED, and EL using chemiluminescence of zymosan-activated polymorphonuclear leukocytes. SDG and vitamin E were used for comparison. The highest antioxidant activity was reported with SECO and ED, whereas, vitamin E resulted in the lowest

antioxidant activity. The antioxidant potency of SECO, ED, EL, and SDG was 4.86, 5.02, 4.35, and 1.27 respectively, compared to vitamin E (Prasad, 2000).

Phytoestrogens, including lignans exhibit both in vitro and in vivo weak estrogenic and antiestrogenic actions (Landete, 2012). Mammalian lignans have an aromatic structure similar to the endogenous estrogen, estradiol (Morris, 2007). It is believed that mammalian lignans act by binding to estrogen receptors (**ER**) on cell membranes (Landete, 2012; Morris, 2007). Enterolactone can function as weak estrogen, in this circumstance EL binds to ER and mimic the action of endogenous estrogen working as an agonist (Landete, 2012). For example, EL can stimulate growth of breast cancer cells as reported by Wang and Kurzer (1997). Anti-estrogenic proprieties of EL have also been reported. In cell-based studies EL binds the ER inhibiting breast cancer cells growth (Mousavi and Adlercreutz, 1992; Buck et al., 2010). In this situation, EL blocks the action of endogenous estrogen, working as antagonists (Landete, 2012).
In addition to the estrogen-like activity of mammalian lignans, studies have also reported that mammalian lignans exhibit effects on hormone metabolism and availability. For example, EL has been shown to stimulate the synthesis of sex hormone binding globulin, which binds sex hormones and reduce their circulation in blood, thus decreasing their biological activity (Thompson et al., 1996). Furthermore, mammalian lignans are believed to influence enzyme activity. For instance, EL inhibit the activity of aromatase, an enzyme involved in the production of estrogens (Landete, 2012, Adlercreutz et al., 1993).

Researches have reported the important role of mammalian lignans in preventing various types of cancer specially the hormone sensitive ones, primarily breast and prostate cancer. In vitro studies have reported that mammalian lignans are possibly responsible for growth inhibition of human prostate cancer (Westcott and Muir, 2003) and breast cancer cells (Buck et al., 2010).

Additionally, epidemiological studies have linked high lignan intake to lower cancer risk. For instance, Touilland et al. (2007) conducted a seven years long study with 58,049 female participants and reported that high dietary intake of plant lignans were associated with reduced risks of postmenopausal breast cancer. More recently, Buck et al. (2010) conducted a meta-analysis to investigate the association between lignans and breast cancer risk. The meta-analysis investigated a total of 21 studies, in which high lignans intake was associated with a significant reduction in breast cancer risk in postmenopausal women (Buck et al., 2010). Despite the potential human health benefits of mammalian lignans, intake of phytoestrogens may also have adverse health effects, particularly in critical stages of infant development (Setchell, 1998; Zung et al., 2008; Landete, 2012) and timing of exposure is crucial to maximize potential health benefits while minimizing adverse health effects.

Improving enterolactone concentration in milk of dairy cows

The concentration of EL in milk of dairy cows can be modulated by dietary changes. Improved concentrations of EL have been reported with feeding SDG-rich sources, such as, FM and flaxseed hulls to dairy cows. Petit et al. (2009b), conducted a study to determine the effects of feeding FM and whole flaxseed (both fed at 10% of DM) on concentrations of ED and EL in milk of Holstein cows. The mammalian lignan ED was not detected in milk. Milk enterolactone concentration was higher in cows receiving FM (0.713 mg/d of EL in milk) than those fed whole flaxseed (0.505 mg/d) and the control diet (no flaxseed products, 0.231 mg/d). Feeding the two flaxseed products resulted in different intakes of SDG: 15280 mg/d in cows receiving FM and 8050 mg/d in cows receiving whole flaxseed which is explain by the fact that lignans are concentrated in the outer fibre containing layers of grains (Adlercreutz & Mazur, 1997) which

leads to higher SDG concentration in flax products with lower concentrations of oil. Lower SDG intake in cows receiving whole flaxseed explains the lower EL concentration in milk reported in this study in cows fed whole flaxseed at 10% of the DM (Petit et al., 2009b). Petit and Gagnon (2009b) fed Holstein cows with incremental amounts of FM: 0, 5, 10 and 15% of DM and reported that the concentration of EL in milk increased linearly. Concentration of EL in milk, expressed as mg/d, was 0.175, 0.312, 0.393 and 0.535 for cows receiving 0, 5, 10 and 15% of FM, respectively. Concentration of ED in milk was below detection level. Similarly, Petit and Gagnon (2011) reported linear increase on milk EL concentration with feeding increasing levels of flaxseed hulls (0, 5, 10, 15, 20% of DM) to Holstein cows (Petit and Gagnon, 2011).

Lima et al. (2016) investigated the effects of dietary FM and abomasal infusion of flaxseed oil and their interaction on milk enterolactone concentration in rumen-fistulated Holstein cows. Cows received four different diets: (1) control diet with no FM (CON); (2) diet containing 12.4 % of FM in the dry matter; (3) no FM and 250 g of flaxseed oil/day infused in the abomasum; and (4) 12.4% of FM and 250 g flaxseed oil/day infused in the abomasum. Dietary FM increased concentrations of EL in milk. Milk EL concentration were 2.11 mg/d and 2.61 mg/d in cows fed 12.4% of FM and no oil (diet 2) and those fed 12.4% of FM and flaxseed oil infusion in the abomasum (diet 4), respectively (Lima et al., 2016).

Recently, it was reported that the concentration of EL in milk can be modified by the type of NSC source supplemented to dairy cows fed diets containing FM, with LM resulting in greater milk EL than GRC (Brito et al., 2015). In this study, Jersey cows were fed mixed mostly grass hay and one of the following 4 concentrate blends: (1) GRC (12% of DM) plus a protein mix containing soybean meal (11% of DM) and sunflower meal (5% of DM); (2) GRC (12% of DM) plus flaxseed meal (16% of DM); (3) LM (12% of DM) plus the same protein mix of diet 1; or

(4) LM (12% of DM) plus flaxseed meal (16% of DM). Milk EL concentration were: 0.37, 1.68, 0.75 and 2.40 mg/d in cows fed diets 1, 2, 3 e 4, respectively. Increased milk EL concentration was observed when FM was fed to the cows. Additionally, it was reported that cows fed LM and FM had higher concentration and yield of milk EL than those fed GRC and FM. This finding suggests that LM, which is a sucrose source, may be more efficient in selecting for ruminal microbes with higher capacity to metabolize plant lignans to mammalian lignans in the rumen than GRC, which is a starch source (Brito et al., 2015).

Considering that *Prevotella spp*. may play a role in lignan metabolism in dairy cows (Schogor et al., 2014), dietary changes that favor or result in a greater prevalence of *Prevotella spp.* in the rumen could improve EL secretion on milk. Li et al. (2015) reported that when steers were fed diets containing 4% flaxseed oil the genus *Prevotella* dominated the ruminal bacterial community, suggesting that PUFA supplementation favors *Prevotella spp.* growth in the rumen. Despite these findings, the effect of PUFA on SDG metabolism and subsequent milk EL concentration has not been fully investigated.

Replacing starch with sugars

There has been much interest in feeding sugars to dairy cattle. Dietary starch has been replaced by sucrose in dairy cow diets with variables results on animal performance and milk composition. Broderick and Radloff (2004) conducted 2 studies to investigate the effects of replacing corn (a source of starch) with molasses (a source of sugars, primarily sucrose) in lactating dairy cows receiving diets based on alfalfa and corn silage. In their first experiment, high moisture shelled corn was replaced by incremental amounts of dried molasses (0, 4, 8 and 12% of diet DM) representing 2.6, 4.2, 5.6 and 7.2% total dietary sugars, respectively. In their

second trial, high moisture shelled corn was replaced by increasing amounts of LM (0, 3, 6 and 9 % of diet DM) representing 2.6, 4.9, 7.4 and 10% total dietary sugars, respectively. They demonstrated that replacing high-moisture shelled corn by incremental amount of dried molasses increased dry matter intake (**DMI**) linearly, whereas quadratic and cubic effects were observed for DMI when corn was substituted by increasing levels of LM. Increased DMI with feeding sugars may be associated with improved palatability according to previous research (Broderick and Radloff, 2004; Broderick et al., 2008).

Regarding the effects of substituting corn with molasses on production, Broderick and Radloff (2004) reported that milk yield responded cubically or quadratically in dairy cows fed incremental amounts of dried molasses and LM, respectively. Furthermore, it was observed that yield of 3.5% fat-corrected milk (**FCM**) tended to respond quadratically or decreased linearly with feeding dried molasses or LM, respectively (Broderick and Radloff, 2004). These authors concluded that the overall optimum for total dietary sugars based on yields of milk and milk components was approximately 5% which correspond to 2.4% added sugars. Feeding > 6% total sugars resulted in depressed milk production (Broderick and Radloff, 2004).

In a subsequent study, Baurhoo and Mustafa et al. (2014) reported no effects on yields of milk, 4% FCM, and energy-corrected milk (**ECM**) when replacing high-moisture corn by increasing amounts of dried molasses (0, 3, and 6% of the diet DM) in dairy cows fed alfalfa silage-based diets. Brito et al. (2015), working with Jersey cows, reported no effect on DMI and milk yield when comparing GRC (12% of the diet DM) and LM (12% of the diet DM) as the NSC supplemental sources, but both 4% FCM and ECM yields decreased in cows receiving LM. Recently, Brito et al. (2017) reported that yields of milk, 4% FCM, and ECM were not changed in grazing Jersey cows fed (12% of the diet DM) GRC or LM as the NSC supplemental sources.

Broderick et al. (2008) conducted a study to investigate the effects replacing starch from corn with sucrose on dairy cows performance. Cows were fed four diets with different corn and sugar cocentrations: 1) 7.5% corn starch, 0% sucrose; 2) 5.0% starch, 2.5% sucrose; 3) 2.5% starch, 5.0% sucrose; or 4) 0% starch, 7.5% sucrose. Substituting corn starch with increasing levels of sucrose increased DMI linearly as observed in the previous study (Broderick and Radloff, 2004). However, no effects were reported for milk yield when corn was replaced with sucrose. Additionally, the authors reported linear decreases in both feed (milk/DMI) and milk N (milk N/N intake) efficiencies when sucrose replaced dietary starch. There was no effect of sucrose addition on nutrient utilization when feed efficiency was expressed as FCM yield/DMI or solids-corrected milk yield/DMI (Broderick et al., 2008).

There have been inconsistent results in milk composition with replacing corn with molasses. In the study conducted by Broderick and Radloff (2004) quadratic and cubic effects were reported for yields of milk fat and protein, respectively, in cows fed increasing amounts of dried molasses, and linear, quadratic, and cubic responses for the concentration and yield of milk protein when feeding incremental levels of LM. Broderick et al. (2008) reported linear increases in milk fat content and secretion with elevated dietary sucrose. Acetate and butyrate are important precursors for de novo fatty acid synthesis in the mammary gland of cows (Bauman and Griinari, 2001). Increased fat secretion due to sugar feeding has been attributed to elevated butyrate production in the rumen (Murphy, 1999). However, increasing dietary sucrose did not change the concentrations of butyrate and total volatile fatty acids (**VFA**) in the study done by Broderick et al. (2008) and the authors stated that the increased fat secretion when sucrose replaced dietary starch was mediated by the increased energy intake. In contrast, substituting high-moisture corn for increasing amounts of dried molasses did not change milk composition

(fat, protein, and lactose) in the study of Baurhoo and Mustafa (2014). When LM was fed as the sole supplemental NSC to Jersey dairy cows fed hay-based diets yield of milk fat was depressed (Brito et al., 2015). However, feeding LM in pasture-based diets did not change the concentrations and yields of milk fat, protein, and lactose in the study done by Brito et al. (2017).

In summary, these results collectively suggest that differences in the ingredient composition of the basal diet, level of molasses fed, forage source, and forage-to-concentrate ratio among other factors may be responsible for the inconsistent results on performance and milk composition in dairy cows across the literature.

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Table 1. Nutritional composition of whole flaxseed (Kajda et al., 2015)

Nutrients	Amount per 100 g of edible flaxseed
Moisture, g	6.5
Protein, g	20.3
Fat, g	37.1
Minerals, g	2.4
Crude fiber, g	4.8
Total dietary fiber, g	24.5
Carbohydrates, g	28.9
Energy, kcal	530.0
Potassium, mg	750.0
Calcium, mg	170.0
Phosphorus, mg	370.0
Iron, mg	2.7
Vitamin A, µg	30
Vitamin E, mg	0.6
Thiamine (B_1), mg	0.23
Riboflavin (B_2), mg	0.07
Niacin, mg	1.0
Pyridoxine, mg	0.61
Pantothenic acid, µg	0.57
Biotin, µg	0.6
Folic acid, µg	112

Table 2. Chemical composition of flaxseed meal, canola meal, soybean meal and cottonseed meal. Value are expressed as % DM except amino acids, which are expressed as % crude protein (Valadares Filho et al., 2006)

Item	Protein Supplements			
	Flaxseed meal	Canola meal	Soybean meal	Cottonseed meal
DM	91.6	90.1	88.6	90.2
CP	34.3	40.0	48.8	35.0
EE	1.32	1.32	1.71	1.38
NDF	25.0	30.7	14.6	28.5
ADF	16.4	21.77	9.86	28.8
Lysine	3.85	2.36	2.82	1.45
Methionine	1.86	0.83	0.63	0.62
Cysteine	1.56	1.02	0.66	0.51
Threonine	3.65	1.67	1.8	1.27
Tryptophan	1.66	0.48	0.67	0.55
Phenylalanine	4.93	1.56	2.34	2.00
Leucine	6.00	2.69	3.62	2.21
Isoleucine	4.18	1.41	2.07	1.11
Valine	4.99	1.64	2.16	1.64
Histidine	2.15	1.00	1.16	1.00
Arginine	9.10	3.94	5.5	3.94
Tyrosine	2.71	1.21	1.5	1.21
Alanine	4.50	1.64	2.06	1.64
Aspartate	9.14	3.17	5.50	3.17
Glutamate	18.3	7.10	8.70	7.10
Glycine	5.84	1.64	1.97	1.64
Serine	3.88	1.66	2.47	1.66

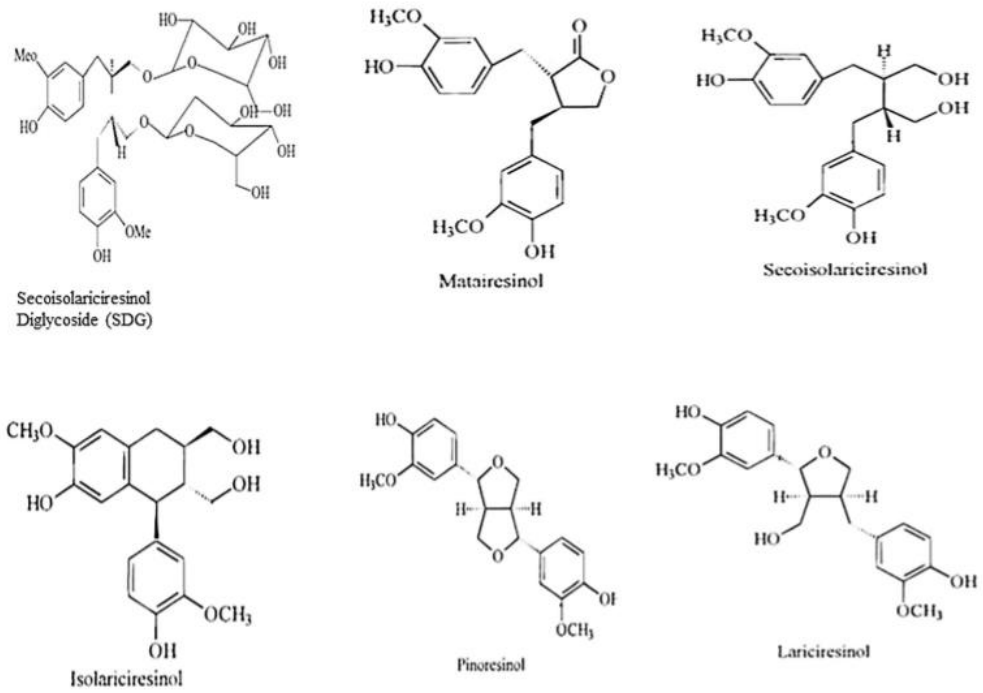

Figure 1. Chemical structure of flaxseed lignans (Adapted from Landete, 2012 and Kajla et al., 2015).

Table 3. Lignan content of flaxseed (adapted from Muir, 2006 and Thompson et al.,2006)

Serving Size	SDG[1]	MAT[2]	LAR[2]	PINO[2]	SECO[2]	Total lignans[2]
mg/100 g	82-2600	0.15	2.8	0.7	375	379
mg/ one tbsp. (11g) of whole seed	11-286	0.02	0.3	0.1	41	42
mg/ One tbsp. of milled flax (8 g)	8-208	0.01	0.2	0.1	30	30

Abreviations: LAR: lariciresinol; MAT: matairesinol; PINO: pinoresinol.
[1] data adapted from Muir, 2006;
[2] data adapted from Thompson et al., 2006. The values for total lignans were calculated by summing the values for MAT, LAR, PINO and SECO.

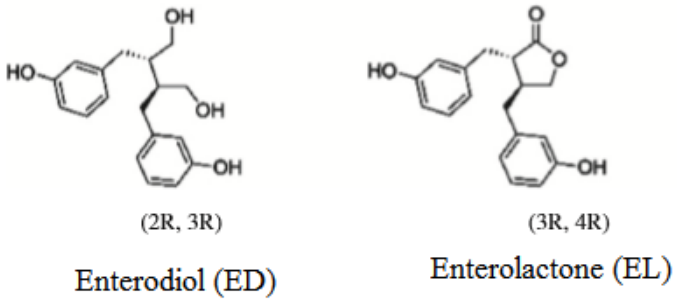

Figure 2. Chemical structure of the mammalian lignans (Landete, 2012).

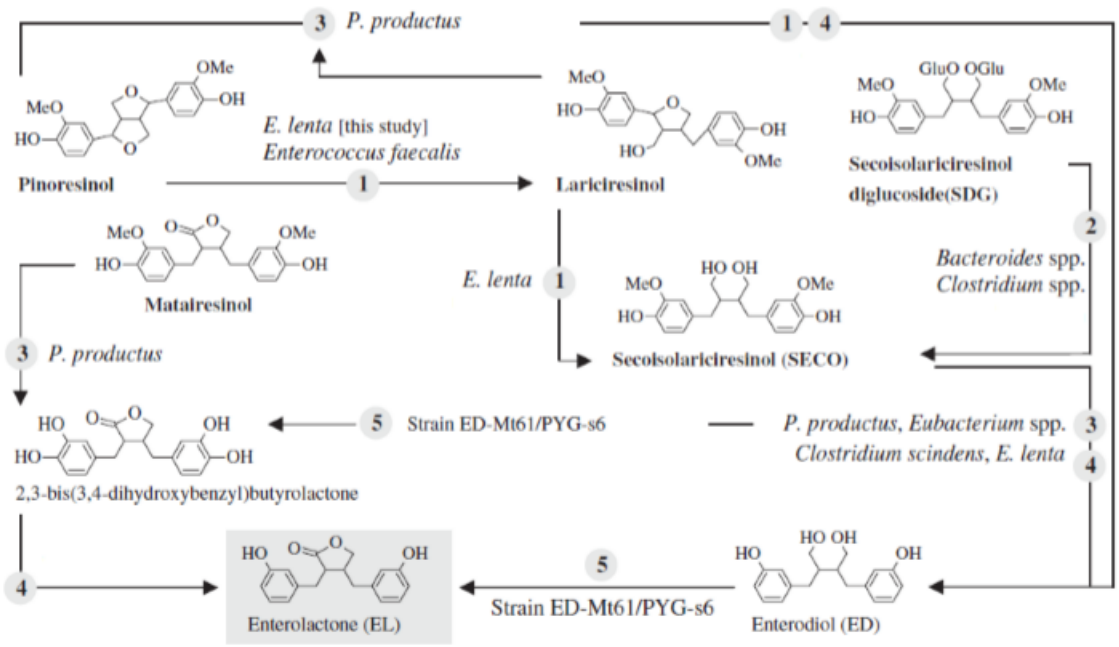

Figure 3. Pathways for mammalian lignans synthesis from plant lignans. Numbers indicate the reactions catalyzed by intestinal bacteria in humans: (1) indicates reduction reaction, (2) deglycosylation, (3) demethylation, (4) dihydroxylation, and (5) dehydrogenation (adapted from Clavel et al., 2006).

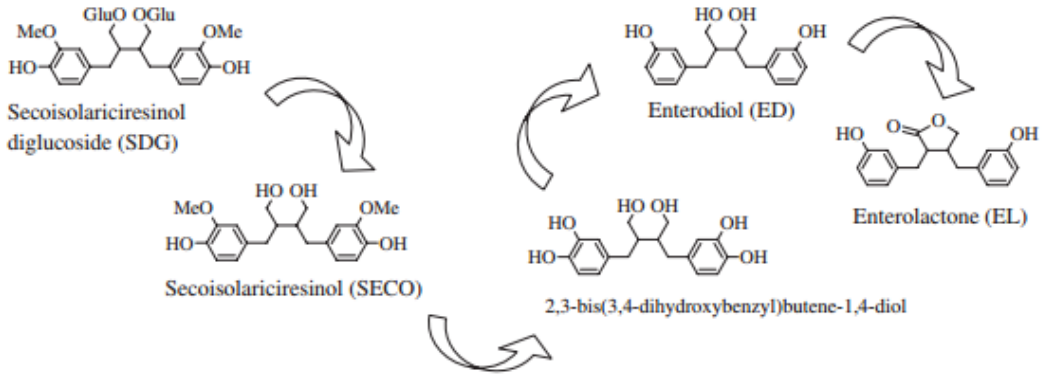

Figure 4. Metabolism of SDG to mammalian lignans (Clavel et al., 2006).

CHAPTER II: REPLACING GROUND CORN WITH INCREMENTAL AMOUNTS OF LIQUID MOLASSES DOES NOT CHANGE MILK ENTEROLACTONE BUT DECREASES PRODUCTION IN DAIRY COWS FED FLAXSEED MEAL

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Replacing ground corn with incremental amounts of liquid molasses does not change milk enterolactone but decreases production in dairy cows fed flaxseed meal. C.P. Ghedini; D.C. Moura; A.S. Oliveira; R. A. Santana; A.F. Brito. https://doi.org/10.3168/jds.2017-13689. J Dairy Sci. 2018 (in press).

INTRODUCTION

Lignans are polyphenolic, phytoestrogenic compounds known to elicit a wide range of biological activities including weak estrogenic and cardioprotective effects, as well as antiestrogenic, antioxidant, anti-inflammatory, and anticarcinogenic properties (Adolphe et al., 2010; Högger, 2013; Imran et al., 2015). Flaxseed (*Linum usitatissimum* L.) is the richest source of the lignan secoisolariciresinol diglycoside (**SDG**), which is a precursor for the synthesis of the mammalian lignans enterolactone (**EL**) and enterodiol by the gut microbiota of humans (Thompson et al., 1991; Gaya et al., 2016) and ruminants (Gagnon et al., 2009; Zhou et al., 2009). Feeding incremental amounts of flaxseed meal (**FM**) to dairy cows increased linearly the milk concentration of EL, whereas no enterodiol was detected in milk (Petit and Gagnon, 2009). Recently, we reported that the concentration of EL in milk was modified by the type of NSC source supplemented to dairy cows fed diets containing (DM basis) 15% FM, with liquid molasses (**LM**) leading to greater milk EL than ground corn (**GRC**) (Brito et al., 2015). This suggests that compared with GRC, LM may select for ruminal microorganisms with better capacity to convert FM-SDG to EL. However, we are not aware of any study to date that has investigated whether milk EL concentration behaves in a linear or curvilinear fashion in response to incremental amounts of LM in cows offered FM. There is also lack of information regarding

whether LM could interact synergistically with GRC to maximize the concentration of EL in milk.

There has been a continuous interest in the use of sugarcane molasses in both conventional (Broderick and Radloff, 2004; Martel et al., 2011; Siverson et al., 2014) and organic (Soder et al., 2012; Brito et al., 2015; Brito et al., 2017) dairy systems in the United States. Whereas conventional dairy cows have been fed dried molasses or LM accompanied by other NSC supplements and corn silage (Broderick and Radloff, 2004; Martel et al., 2011; Siverson et al., 2014), organically-certified dairy cows are usually fed LM as the sole NSC supplemental source to grass hay (Brito et al., 2015) or mixed grass-legume pasture (Soder et al., 2012; Brito et al., 2017). Previous dose-response studies resulted in inconsistent responses on DMI, milk yield and composition, and nutrient digestibility when dried or LM partially replaced high-moisture corn (Broderick and Radloff, 2004; Baurhoo and Mustafa, 2014) or GRC (Martel et al., 2011) in moderate- to high-starch diets. However, research addressing the impact of completely replacing GRC with incremental amounts of LM on production, milk composition, and nutrient utilization in dairy cows fed mixed grass-legume baleage and low-starch diets is lacking.

We hypothesized that: (1) the concentration of EL in milk would be modulated by expected changes in DM and SDG intakes when replacing GRC with incremental amounts of LM in cows fed FM. We also hypothesized that replacing GRC with LM would lead to: (2) increased milk yield and milk N efficiency (i.e., milk N/ N intake) due to improved balance between RDP and fermentable energy supply as sucrose from LM is more rapidly degraded in the rumen than starch from GRC; and (3) marked changes in milk fatty acid (**FA**) profile in response to expected differences on 18-C FA intake between GRC and LM. The objective of this

study was to evaluate the effects of replacing GRC with incremental amounts of LM on milk EL concentration, antioxidant enzymes activity in plasma, production, milk FA profile, and apparent total-tract digestibility of nutrients in Jersey cows fed FM and low-starch diets.

MATERIALS AND METHODS

This 84-d long study was conducted at the University of New Hampshire Burley-Demeritt Organic Dairy Research Farm (43°10'N, 70°99'W; Lee, NH) from December 3, 2014 to February 24, 2015. Care and handling of cows used in our study were conducted as outlined in the guidelines of the University of New Hampshire Institutional Animal Care and Use Committee (IACUC Protocol# 140901).

Cows, Experimental Design, and Diets

Sixteen multiparous organically-certified Jersey cows averaging (mean ± standard deviation) 101 ± 45 DIM, 462 ± 38 kg BW, and 19.8 ± 3.90 kg/d of milk in the beginning of the study were used. Distribution of cows to squares was done to balance for differences in DIM (square 1 = 163 ± 10 DIM; square 2 = 112 ± 16 DIM; square 3 = 78 ± 7 DIM; square 4 = 50 ± 10 DIM). Within each square, cows were randomly assigned to treatment sequences in a replicated 4 × 4 Latin square design. Each experimental period lasted 21 d with 14 d for diet adaptation and 7 d for data and sample collection. The experimental diets were fed as TMR consisting (DM basis) of 31.2% mixed-mostly grass baleage, 20.8% mixed-mostly legume baleage, 8% grass hay, 8.5% soyhulls, 2.5% roasted soybean, 15% FM, and 2% minerals-vitamins premix. The GRC:LM dietary ratios (DM basis) were 12:0, 8:4, 4:8, and 0:12. Sugarcane LM was purchased from Buffalo Molasses, LCC (North Java, NY). The nutritional composition of the feedstuffs

used in this study is presented in Table 4, and the ingredient and nutritional composition of the experimental diets are reported in Table 5.

Cows were housed in a bedded-pack barn with dried pine shavings as bedding and kept in a pen separated from the remaining lactating cows in the herd. The bedding area (132 m^2) opens to a 478-m^2 concrete-floor outdoor lot (total pen area = 610 m^2) allowing cows to walk freely in compliance with the USDA National Organic Program "Livestock living conditions" (section 205.239; https://www.ecfr.gov/cgi-bin/text-idx?SID=38fb700cb79331adaec0a1e05477134f&mc=true&node=se7.3.205_1239&rgn=div8; accessed August 4, 2017), which calls for year-round access to the outdoors for all ruminants among other regulations. Cows had access to a roof-covered feeding station equipped with electronic recognition Calan doors system (American Calan Inc., Northwood, NH) located at the end of the pen.

Feeding Management and Feed Sampling and Analyses

All bales used in this study were sampled prior to feeding using a Hilti model TE 7-A drill (Hilti North America, Tulsa, OK) fitted with a 40-cm long metal core sampler. Throughout the study, approximately 100-g forage samples were obtained after 3 to 4 core samplings from each bale and dried in a forced-air oven set at 55°C for approximately 48 h with the resulting DM used to adjust the daily proportions of forages in the as-fed TMR. Baleage and hay bales were chopped using a TMR vertical mixer (Valmetal V-Mix 400; Saint-Germain-de-Grantham, QC, Canada). All dietary ingredients, including LM, were mixed twice daily using an A100 Self-Propelled mixer (Jaylor Fabricating Inc.; East Garafraxa, ON, Canada) and offered to the cows as TMR at 0700 and 1600 h. Approximately 40% of the daily TMR allocation was offered in the

morning and the remaining 60% in the afternoon to account for uneven intervals between feeding times (i.e., 9 h between 0700 h and 1600 h and 15 h between 1600 h and 0700 h). The amount of each dietary ingredient used to make the TMR was recorded using the A100 Self-Propelled mixer (Jaylor Fabricating Inc.), which is equipped with an electronic scale system (Dinamica Generale US Inc., St. Charles, IL). Refusals were collected daily before the afternoon feeding and weighed as done for the TMR. The amount of TMR offered to the cows was adjusted daily to allow refusals of 5 to 10% of the as fed intake. The Calan doors system (American Calan Inc.) was used to individualize the dietary treatments and feed intake was recorded by subtracting the total amount of TMR offered daily from that of refusals. Body weight was measured immediately after the afternoon milking during 3 consecutive days before the beginning of the study and during the last 3 d of each sampling week to determine BW change.

Samples of TMR, feeds, and refusals (composited by diet) were collected daily during each sampling week, composited by period, dried in a forced-air oven (55°C, 48 h), and ground to pass through a 1-mm screen (Wiley mill; Arthur H. Thomas, Philadelphia, PA). Feeds (baleages, hay, soyhulls, GRC, roasted soybean, soybean meal, and FM) were shipped to a commercial laboratory (Dairy One Cooperative Inc., Ithaca, NY) and analyzed for: DM (method 930.15; AOAC International, 2016), total N (method 990.03; AOAC International, 2016), NDF (method 6 in an ANKOM^{200} Fiber Analyzer with α-amylase and sodium sulfite; ANKOM Technology, Fairpoint, NY; solutions as in Van Soest et al., 1991), ADF [method 5 in an ANKOM^{200} Fiber Analyzer; solutions as in method 973.18 (AOAC International, 2016)], acid detergent lignin [method 9 in a ANKOM Daisy Incubator; solutions as in method 973.18 (AOAC International, 2016)], ether extract [extraction by a Soxtec HT6 System (Foss North America, Eden Prairie, MN) using anhydrous diethyl ether; method 2003.05 (AOAC International, 2016)],

starch (YSI 2700 Select Biochemistry Analyzer, application note no. 319; YSI Inc. Life Sciences, Yellow Springs, OH), water-soluble carbohydrates [(**WSC**); Hall et al., 1999], and ash (method 942.05; AOAC International, 2016). Liquid molasses was analyzed (Dairy One Cooperative Inc.) for DM, total N, ether extract, ash, and WSC, while refusals were analyzed for NDF and ADF in addition to DM, total N, and ash using the procedures reported above. Individual minerals (Ca, P, Mg, K, Na, Fe, Zn, Cu, Mn, Mo, and S) were analyzed (Dairy One Cooperative Inc.) in all feed samples using an iCAP 6300 Intrepid Inductively Coupled Plasma Radial Spectrometer (Thermo Fisher Scientific Inc., Waltham, MA) after microwave digestion (CEM application note for acid digestion; CEM, Matthews, NC). All feeds were also analyzed (Analab, Fulton, IL) for individual sugars using a Beckman HPLC (Beckman Coulter Inc., Fullerton, CA) equipped with an evaporative light scattering detection system. Samples of TMR were sent to the Pennsylvania State University (University Park, PA; Kevin Harvatine Laboratory) for FA analysis using GLC after direct methylation (Sukhija and Palmquist, 1988). Flaxseed meal samples were shipped to Bioprofile Testing Laboratories (St. Paul, MN) for SDG analysis using HPLC according to the procedures described by Muir and Westcott (2000).

Milk Sampling and Analyses

Cows were milked twice daily at approximately 0500 and 1530 h in a 4-stall step-up parlor equipped with headlocks (Agromatic; Fond DuLac, WI), automatic take-offs, and milk meters (Westfalia Surge; GEA Farm Technologies Inc., Naperville, IL). Milk weights were recorded daily throughout the duration of the experiment (DairyPlan C21 Version 5.2; GEA Farm Technologies Inc.). Milk samples were collected during 4 consecutive milkings (d 20 and 21) of each sampling week, stored in tubes containing 2-bromo-2-nitropropan-1,3 diol, pooled by

cow according to morning and evening milk weights, and refrigerated at 4°C until shipped to Dairy One Cooperative Inc. for determination of fat, true protein, lactose, and MUN by Fourier transform infrared spectroscopy using a MilkoScan FT+ (Foss Inc., Hillerød, Denmark) and SCC by flow cytometry in a Fossomatic FC (Foss Inc.). Subsamples of milk without preservative were collected concurrently, pooled using the same procedure described above, and stored at -20°C until analyzed for EL and milk FA. Milk EL was extracted and hydrolyzed [β-glucuronidase/arylsulfatase from *Helix pomatia* (Roche-Diagnostics; Laval, QC, Canada)] according to procedures described previously (Gagnon et al., 2009). After extraction and hydrolysis, EL was analyzed colorimetrically in quadruplicate using a competitive commercial enzymatic immunoassay (assay kit no. 500520; Cayman Chemical Co., Ann Arbor, MI) that recognizes both enantiomeric forms of EL in an UV/visible spectrophotometer (Beckman Coulter Inc., Brea, CA) set at a wavelength of 405 nm. Milk samples were shipped to the Pennsylvania State University (University Park, PA; Kevin Harvatine Laboratory) for FA analysis using GLC according to Rico and Harvatine (2013).

Blood sampling and Analyses

Blood samples were collected from the coccygeal vein or artery approximately 4 h after the morning feeding on d 21 of each sampling week using 10-mL vacutainer tubes containing K_3-EDTA (Covidien, Minneapolis, MN). After collection, tubes were kept on ice until processing. Samples were centrifuged (3,300 × *g*, 20 min, 4°C) and 2 aliquots of plasma were collected. The first aliquot was stored at -80°C for determination of antioxidant enzymes activity, while the second one was stored at -20°C for determination of plasma urea N (**PUN**) concentration. After thawed in room temperature, plasma samples were analyzed

colorimetrically with a UV/Vis spectrophotometer (Beckman Coulter Inc.) for PUN (diacetyl-monoxime method; Rosenthal, 1955) and catalase (assay kit # 707002; Cayman Chemical Co.) with wavelength set a 540 nm, and for superoxide dismutase [(**SOD**); assay kit # 706002; Cayman Chemical Co.] and glutathione peroxidase [(**GPx**); assay kit no. 703102; Cayman Chemical Co.] at wavelengths of 450 and 340 nm, respectively.

Fecal and Urinary Sampling and Analyses

Fecal and urinary samples were collected once daily on d 19 (0700 h), d 20 (1200 h), and d 21 (1800 h) during each sampling week. Fecal grab samples were collected by stimulating defecation or directly from the rectum. After collection, fecal samples were stored in plastic bags at -20°C and later dried in a forced-air oven (55°C, 72 h). Next, samples were ground to pass through a 1-mm screen (Wiley mill) and pooled by cow based on dry weight over the 3 d for obtaining a single composite sample/cow per period. Fecal samples were analyzed for DM, ash, total N, NDF, ADF, and starch as described previously. Approximately 0.5 g of feces, feeds, and TMR was weighed into Ankom F57 bags (Ankom Technology), placed in 1 larger laundry bag, and inserted in the rumen of 1 ruminally-cannulated lactating Jersey cow for 12 d. After removal from the rumen, bags were rinsed with tap water and analyzed for ADF as described earlier. Indigestible ADF was used as an intrinsic marker to estimate fecal output of DM and apparent total-tract digestibility of nutrients (Cochran et al., 1986; Huhtanen et al., 1994).

Spot samples of urine were collected concurrently with fecal samples by stimulation of the pudendal nerve massaging the area bellow the vulva. After collection, urinary samples were immediately transported to the laboratory for processing. Subsamples of urine from each cow (approximately 2.7 mL/time point) were pooled over 3 d into 50-mL centrifuge tubes containing

32 mL of 0.072 *N* H_2SO_4 and stored at -20°C until analysis. Urinary samples were thawed at room temperature and analyzed colorimetrically for creatinine (assay kit no. 500701; Cayman Chemical Co.), allantoin (Chen et al., 1992), uric acid (assay kit no. 1045-225; Stanbio Laboratory, Boerne, TX), and total N (micro-Kjeldahl analysis; AOAC, 2016; Dairy One Cooperative Inc.). Allantoin and uric acid were read at wavelengths of 522 and 520 nm, respectively, on a UV/visible spectrophotometer (Beckman Coulter Inc.). Daily urinary volume was estimated from the urinary concentration of creatinine assuming a constant creatinine excretion rate of 0.212 mmol/kg of BW (Chizzotti et al., 2008). Urinary excretion of allantoin, uric acid, total purine derivatives (allantoin + uric acid), total N, and urea N were calculated by multiplying the urinary volume by the concentration of each metabolite in urine.

Statistical Analyses

Data were analyzed using the MIXED procedure of SAS (SAS version 9.4; SAS Inst. Inc., Cary, NC) according to a replicated 4 × 4 Latin square design. Squares were balanced for potential first-order carryover effects in subsequent periods (Williams, 1949; Kim and Stein, 2009) as each treatment immediately preceded and followed every other exactly once in each square. The following model was fitted for all variables:

$Y_{ijkl} = \mu + S_i + P_j + C_{k(i)} + T_l + S \times T_{il} + E_{ijkl}$,

where Y_{ijkl} = dependent variable, μ = overall mean, S_i = fixed effect of ith square, P_j = fixed effect of jth period, $C_{k(i)}$ = random effect of kth cow within ith square, T_l = fixed effect of lth treatment, $S \times T_{il}$ = interaction between ith square and lth treatment, and E_{ijkl} = error term. Orthogonal polynomials were used to test linear, quadratic, and cubic effects in response to incremental dietary levels of LM in cows fed FM. All reported values are least square means ±

standard error of the mean. Significance was declared at $P \leq 0.05$ and trends at $0.05 < P \leq 0.10$. Milk concentration of EL was transformed (natural log) before statistical analyses, but results presented herein were reported as adjusted mean values of quadruplicate runs on the original scale of measurements as done previously (Petit and Gagnon, 2009; Brito et al., 2015).

RESULTS AND DISCUSSION

Feeds and Diets Nutritional Composition

Sucrose was the predominant sugar found in LM accounting for 77% of the total individual sugars measured chromatographically (Table 4). The concentrations of sucrose and total individual sugars in LM fed in the present study were similar to those obtained by Brito et al. (2017), but 38 and 49% lower, respectively, than values reported by Brito et al. (2015), thereby showing substantial variation in sugars content from source to source. As expected, the concentration of WSC, which was measured colorimetrically, was greatest in LM (Table 4). On average, the concentration of total individual sugars in LM analyzed chromatographically was 39% lower than that analyzed colorimetrically (Table 4), indicating that the analytical method used should be taken into account when making recommendations about optimum dietary levels of LM in dairy diets. The concentration of SDG in FM averaged 1.8% and was similar to the SDG content (mean = 1.66%) reported by Brito et al. (2015).

The dietary concentration of starch decreased from 9.6 to 1.64% and that of added individual sugars and WSC provided by LM increased from 0 to 4.95% and 0 to 8.08%, respectively, when replacing GRC by incremental amounts of LM (Table 5). Total individual sugars and total WSC in the experimental diets increased from 3.90 to 8.63% and 4.02 to 12.9%,

respectively, with replacing GRC for increasing levels of LM (Table 5). The dietary concentrations of CP (mean = 18.8%), NDF (mean = 43.5%), ADF (mean = 28.6%), and NE_L (mean = 1.56 Mcal/kg of DM) were similar across diets (Table 5).

Milk Enterolactone and Plasma Antioxidant Enzymes

Only a cubic trend ($P = 0.08$; Figure 5) was observed for milk EL despite the linear decrease in SDG intake when GRC was substituted with incremental amounts of LM in cows fed 15% FM (Table 6), thus indicating no dietary effect on the content of EL in milk. Even though this cubic trend is difficult to explain biologically, the lack of a precursor-product relationship suggests that the ruminal output of EL seems to be more affected by the microbiota metabolism of SDG than by SDG supply. Milk EL yield did not differ and averaged 1.38, 1.61, 1.36, and 1.52 mg/d when feeding 0, 4, 8, or 12% LM, respectively (data not shown). We reported previously that compared with GRC, LM increased the concentration and yield of milk EL in dairy cows fed grass hay plus 15% of the diet DM as FM (Brito et al. 2015), suggesting that LM may select for ruminal microorganisms with increased capacity to convert SDG to EL. Schogor et al. (2014) reported that *Prevotella* spp. were one of the main converters of SDG to secoisolariciresinol, a lignan-derived metabolite that is further metabolized to enterodiol and EL presumably by other ruminal bacteria species. *Prevotella* species are also capable of utilizing starch, other noncellulosic polysaccharides, and simple sugars as energy sources yielding succinate as the major end product of ruminal fermentation (Purushe et al., 2010). However, ruminal samples were not collected in the present study, which limits our capacity to better understand the relationships among SDG supply, microbiota profile, and EL outputs in rumen and milk.

The plasma activities of the antioxidant enzymes GPx (mean = 119 nmol/min per mL) and catalase (mean = 25.8 nmol/min per mL) did not differ across treatments, while plasma SOD activity tended ($P = 0.06$) to respond cubically when replacing GRC by increasing amounts of LM in diets containing 15% FM (Table 6). Despite similar cubic trend patterns observed for milk EL concentration and plasma SOD activity, the lack of significant effects in response to dietary changes for these 2 variables precludes any speculation about the capacity of EL to modulate the activity of antioxidant enzymes as shown previously (Rajesha et al., 2006; Côrtes et al., 2012). Brito et al. (2015) also observed no effects of FM (15% of the diet DM) on the plasma activities of SDG and GPx in dairy cows fed GRC or LM as the sole supplemental NSC source.

Production and Nutrient Digestibility

Dry matter intake decreased linearly with replacing GRC by incremental amounts of LM in diets containing 15% FM (Table 6). This drop in DMI may have been caused by excess sugars intake and the potential negative impact on ruminal fermentation processes including decreased fiber digestibility. Broderick and Radloff (2004) reported a linear increase for DMI when high-moisture shelled corn was replaced (DM basis) by increasing amounts of dried molasses (0, 4, 8, and 12%), and quadratic and cubic effects with increased LM (0, 3, 6, and 9%). They (Broderick and Radloff, 2004) concluded that the overall optimum for total dietary sugars based on DMI and yields of milk and milk components was approximately 5% (2.4% added sugars). In the present study, the dietary concentrations of added individual sugars and WSC exceeded the 2.4-% threshold at 8 and 4% LM inclusion, respectively (Table 5). The 5-% total dietary sugars threshold was surpassed at 4% LM inclusion (Table 5) independent of the analytical method used. There were marked differences in ingredient and nutritional composition comparing diets

fed by Broderick and Radloff (2004) and those fed herein which may help explain the discrepancy in DMI between these 2 studies in response to molasses supplementation. Furthermore, the range in total dietary sugars was wider in the present study (Table 5) than that (from 2.6 to 10%) reported by Broderick and Radloff (2004) despite different analytical methods used in both experiments. It can be also hypothesized that interactions between molasses and ingredients in the basal diet may have impacted DMI and ruminal fermentation processes, with more negative associative effects in low-starch diets based on mixed grass-legume baleage (present study) than in moderate- to- high-starch diets based on alfalfa and corn silage (Broderick et al., 2004). In fact, Martel et al. (2011) demonstrated that feeding molasses (5% of the diet DM) as a substitute for GRC significantly increased ruminal pH and decreased total VFA concentration in late-lactation Holstein cows receiving high-starch diets. Brito et al. (2015) observed no depression on DMI when feeding 12% of the diet DM as LM (7.4% added sugars) to Jersey cows offered a grass hay-based, low-starch diet, thus implying that forage sources may be more involved in triggering negative or positive associative effects between sugars and the basal diet than dietary starch concentration.

Yields of milk, 4% FCM, and ECM all decreased linearly with replacing GRC for incremental amounts of LM in cows fed 15% FM, which are primarily explained by a linear decrease in DMI (Table 3). Broderick and Radloff (2004) reported that milk yield responded cubically or quadratically in dairy cows fed incremental amounts of dried or LM, respectively. They also observed that yield of 3.5% FCM tended to respond quadratically or decreased linearly in cows receiving dried or LM, respectively (Broderick and Radloff, 2004). In contrast, Baurhoo and Mustafa et al. (2014) reported no effects on yields of milk, 4% FCM, and ECM when high-moisture corn was replaced by increasing amounts of dried molasses (0, 3, and 6% of the diet

DM) in dairy cows offered alfalfa silage-based diets. Brito et al. (2015) observed no effect on milk yield when comparing GRC versus LM as the sole NSC supplemental source, but both 4% FCM and ECM yields decreased significantly in LM cows. Recently, we reported that yields of milk, 4% FCM, and ECM were not changed in grazing dairy cows fed GRC or LM as the sole NSC supplemental source (Brito et al., 2017). These results collectively suggest that differences in the ingredient composition of the basal diet, level of molasses fed, forage source, and forage-to-concentrate ratio among other factors may be responsible for the inconsistent responses on yields of milk, FCM, and ECM across the literature. Feed efficiency expressed as milk yield/DMI (mean = 0.98 kg/kg), 4% FCM yield/DMI (mean = 1.18 kg/kg), or ECM yield/DMI (mean = 1.28 kg/kg) was not affected by feeding LM at expense of GRC (Table 6). Similarly, milk N efficiency did not change and averaged 19.9% across treatments.

The concentrations of milk fat and true protein were not affected by dietary levels of LM, whereas lactose content tended to decrease linearly ($P = 0.06$) in cows offered 15% FM (Table 6). Yields of milk fat, true protein, and lactose followed milk yield and decreased linearly (Table 6). There have been inconsistent results in milk component concentrations and yields in response to dried or LM supplementation. Broderick and Radloff (2004) reported quadratic and cubic effects for yields of milk fat and protein, respectively, in cows fed increasing amounts of dried molasses, and linear, quadratic, and cubic responses for the concentration and yield of milk protein when feeding incremental levels of LM. However, Baurhoo and Mustafa (2014) reported no effects on concentrations and yields of milk fat, protein, and lactose with substituting high-moisture corn for different levels of dried molasses. When GRC or LM was fed as the sole supplemental NSC, yield of milk fat decreased in dairy cows fed grass hay (Brito et al., 2015),

while concentrations and yields of milk fat, true protein, and lactose did not change in those fed pasture (Brito et al., 2017).

The concentration of MUN decreased linearly when GRC was replaced by LM (Table 6). Because the dietary concentration of CP was relatively similar across treatments (Table 5), a linear decrease in N intake (Table 6) appears to explain, at least partially, the observed decrease in MUN. Alternatively, Bannink et al. (1999) reported that intake and urinary excretion of K, Na, and N explained, respectively, 85.8 and 89.8% of the variance when regressed against urinary volume in lactating dairy cows. Eriksson and Rustas (2014) observed a positive linear relationship between K intake and urinary volume and a negative linear relationship between K intake and MUN in dairy cows. Spek et al. (2012) showed that feeding incremental amounts of Na to dairy cows increased linearly the urinary volume and total urinary N excretion, and decreased linearly MUN concentration. In the current study, whereas the intake of K (Table 6) and urinary volume (Table 7) increased linearly with substituting GRC by LM, that of Na decreased linearly (Table 6). Our results suggest that K was likely more involved in the linear decrease of MUN than Na, which is not surprising as the difference in K intake was much larger compared with that of Na intake across treatments. According to Spek et al. (2012) and Eriksson and Rustas, 2014), elevated Na and K intake leads to increased renal glomerular filtration rate and a temporary increase in the urinary excretion of urea N, ultimately dropping both MUN and PUN. The concentration of PUN followed that of MUN and tended ($P = 0.07$) to decrease linearly (Table 6).

The apparent total-tract digestibility of starch responded linearly and quadratically, while that of CP decreased linearly with feeding incremental amounts of LM (Table 7). No treatment effects were observed for DM, OM, NDF, and ADF apparent total-tract digestibilities (Table 7).

Despite linear and quadratic effects for starch digestibility, the actual differences across treatments were too small and not biologically significant. It is also important to note that starch digestibility averaged 99.9% among treatments, which is probably explained by the low dietary concentration of starch (mean = 5.7%; Table 2) and consequent low starch intake (data not shown). The impact of replacing corn grain by dried or LM on apparent total-tract digestibility of nutrients is not consistent based on previous research. Broderick and Radloff (2004) reported linear increases for the total-tract digestibilities of DM, OM, NDF, and ADF when feeding incremental amounts of dried molasses, as well as quadratic and cubic effects for NDF and ADF digestibilities in cows offered increasing levels of LM. In contrast, Baurhoo and Mustafa (2014) observed no effect of different dietary levels of dried molasses on apparent total-tract digestibilities of DM, OM, NDF, CP, and gross energy. When GRC or LM was fed as the sole NSC supplemental source to lactating dairy cows, CP digestibility decreased in LM cows, but no treatment effects were observed for DM, OM, NDF, and ADF digestibilities (Brito et al., 2015). Broderick et al. (2008) reported that the apparent ruminal digestibility of N reduced linearly when sucrose was increased from 0 to 7.5% of diet DM at expanse of corn starch, suggesting a similar response in the present study. Alternatively, the linear drop in CP digestibility observed herein may have been caused by decreased dilution of fecal metabolic N due to lowered N intake (Table 6) as suggested by Kauffman and St-Pierre (2001). When fecal N excretion was corrected for metabolic N, which was estimated assuming an excretion rate of 5.5 g/kg of DMI in Jersey cows (Kauffman and St-Pierre, 2001), the linear response in CP digestibility went from significant (Table 7) to a trend ($P = 0.06$; data not shown). Overall, discrepant results across the literature regarding the impact of dried or LM supplementation on apparent total-tract

digestibility of nutrients may be related to differences in dietary levels of sugars among other potential factors as discussed earlier.

The urinary excretion of total N decreased linearly with substituting GRC with increasing amounts of LM, which is consistent with the linear decrease in urinary volume (Table 7). In contrast, no treatment effects were observed when the urinary excretion of total N was expressed as a proportion of N intake (mean = 42%). Similarly, the urinary excretion of urea N expressed in quantity (mean = 121 g/d), as a proportion of total urinary N (mean = 54%), or as a proportion of N intake (mean = 22%) was not affected by treatments (Table 4). The urinary excretion of allantoin (mean = 164 mmol/d) and total purine derivatives (mean = 202 mmol/d) also did not differ significantly among treatments (Table 7). However, the urinary excretion of uric acid tended ($P = 0.08$) to respond cubically in cows fed LM at expense of GRC (Table 7). Interestingly, the urinary excretion of uric acid followed a cubic pattern very similar compared to that observed for milk EL (Figure 5), with cows fed 4% LM showing the greatest values for these 2 variables. Uric acid is a purine derivative used as an indirect marker to estimate microbial protein synthesis, thus consistent with the response in milk EL. It is well established that FM-SDG is converted primarily to EL by the ruminal microbiota in ruminants (Gagnon et al., 2009; Zhou et al., 2009).

Milk FA Profile

Milk proportions of 8:0, 10:0, 12:0, 18:0, and 20:0 decreased linearly, whereas 16:0 increased linearly in cows fed incremental amounts of LM and 15% FM (Table 8). Intake of 18:0 decreased linearly ($P < 0.01$; data not shown), which is consistent with the linear reduction in milk 18:0. Furthermore, Σ 18-C FA also decreased linearly (Table 8), indicating

less uptake of preformed 18:0 from plasma by the mammary gland possibly as a result of reduced 18:0 intake. Similarly, we observed that the milk proportion of 18:0 decreased in dairy cows fed grass hay (Brito et al., 2015) or pasture (Brito et al., 2017) supplemented with LM versus GRC. In contrast, although intake of 16:0 reduced linearly ($P < 0.01$; data not shown), the milk proportion of 16:0 increased linearly (Table 8), suggesting enhanced de novo synthesis of 16:0 in the mammary gland or uptake from plasma. In fact, Σ 16-C also increased linearly in the present study (Table 8). Brito et al. (2015) observed a similar decoupled response between 16:0 intake and milk 16:0 in cows fed LM versus GRC.

Milk proportions of the branched-chain FA *iso* 14:0 and *iso* 16:0 decreased linearly, whereas *iso* 15:0 and *iso* 17:0 increased linearly in cows fed incremental amounts of LM (Table 8). Milk proportion of the odd-chain FA 15:0 increased linearly and quadratically, with the greatest proportion of this FA observed in the diet containing 12% LM (Table 8). Similarly, the proportion of 17:0 in milk increased linearly and tended ($P = 0.07$) to respond quadratically when GRC was substituted for LM. The Σ odd-chain FA followed the milk proportions of 15:0 and 17:0 and increased linearly and quadratically (Table 8). In our previous research, milk proportions of 15:0 and 17:0 were also greater in cows fed LM compared with GRC (Brito et al., 2015; Brito et al., 2017). It is well known that milk odd- and branched-chain FA are originated primarily from ruminal microbiota cells (Fievez et al., 2012). Therefore, data from the present study appear to indicate that LM may favor the growth of ruminal microbes enriched in *iso* 15:0, *iso* 17:0, 15:0, and 17:0, while inhibiting those enriched in *iso* 14:0 and *iso* 16:0.

Milk proportions of *cis*-9 18:1, *cis*-12 18:1, *trans* 6-8 18:1, *trans*-9 18:1, *trans*-12 18:1, *trans*-15 18:1, *cis*-9, *cis*-2 18:2, and Σ *cis*-18:1 FA all decreased linearly, while *trans*-10 18:1

tended ($P = 0.08$) to reduce linearly when cows were fed incremental amounts of LM at expense of GRC (Table 5). Intake of *cis*-9 18:1 ($P < 0.001$) and *cis*-9, *cis*-12 18:2 ($P < 0.01$) decreased linearly (data not shown), thus explaining the linear reductions in milk *cis*-9 18:1 and *cis*-9, *cis*-12 18:2. The *trans*-11/*trans*-10 ratio also decreased linearly, indicating that ruminal biohydrogenation pathways were shifted toward *trans*-11 18:1 rather than *trans*-10 18:1, which agree with Martel et al. (2011) and our previous research (Brito et al., 2017). Although the milk proportion of *cis*-9, *cis*-12, *cis*-15 18:3 increased linearly as LM replaced GRC (Table 5), intake of this FA decreased linearly ($P < 0.01$; data not shown), thus suggesting that *cis*-9, *cis*-12, *cis*-15 18:3 was less biohydrogenated in the rumen. According to Martel et al. (2011), ruminal protozoa may slow down FA biohydrogenation by maintaining an intracellular pool of FA away from biohydrogenating bacteria. However, the literature is inconsistent regarding whether dietary sugar promotes or inhibits growth of ruminal protozoa (Martel et al., 2011), possibly because of heterogeneous nature of these microbes (Oelker et al., 2009). Milk proportion of *cis*-9, *trans*-11 18:2 increased linearly despite a numerical increase in *trans*-11 18:1 with feeding increasing levels of LM (Table 8). The Δ^9-desaturase index for the for the *trans*-11 18:1/*cis*-9, *trans*-11 18:2 pair increased linearly (Table 8), indicating that elevated *cis*-9, *trans*-11 18:2 resulted from endogenous synthesis via the Δ^9-desaturase enzyme using *trans*-11 18:1 as substrate (Griinari et al., 2000).

CONCLUSIONS

The concentration of EL in milk tended to respond cubically with replacing GRC by incremental amounts of LM in cows fed 15% of the diet DM as FM despite a linear decrease in SDG intake, thus contradicting our first hypothesis. Our second hypothesis that production and

milk N efficiency would be improved due to a better balance between RDP and fermentable energy supply when substituting GRC for LM was also rejected. Our third hypothesis was partly confirmed as the linear decreases in *cis*-9 18:1 and *cis*-9, *cis*-12 18:2 in milk were consistent with decreased intake of these FA in cows fed LM at expense GRC. However, a decoupled response between milk *cis*-9, *cis*-12, *cis*-15 18:3 and intake of this FA was also observed. Further research is needed to better understand how FM-SDG is affected by changes in diversity and function of the ruminal microbiome, and how potential interactions between different forage sources and dietary sugars impact production and N utilization in dairy cows.

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Table 4. Nutritional composition of feedstuffs (mean ± SD) used in the experimental diets (% of DM, unless otherwise noted)[1]

	Forage			Concentrate				
Item	Grass hay	Mixed-mostly legume baleage	Mixed-mostly grass baleage	Flaxseed meal	Soyhulls	Roasted soybean	Ground corn	Liquid[2] molasses
DM, % of fresh matter	92.2 ± 1.30	40.3 ± 3.90	46.7 ± 7.30	90.0 ± 0.87	92.6 ± 1.66	93.6 ± 1.01	90.1 ± 2.38	75.1 ± 1.94
CP	10.7 ± 0.67	21.4 ± 0.98	14.9 ± 1.53	38.5 ± 1.82	13.9 ± 1.76	38.3 ± 1.73	9.73 ± 1.08	6.38 ± 1.23
Soluble protein, % of CP	32.8 ± 1.71	63.0 ± 5.10	62.8 ± 5.32	33.8 ± 4.65	30.3 ± 2.87	14.3 ± 1.26	23.3 ± 4.04	NA[3]
NDF	65.1 ± 1.47	43.2 ± 1.42	59.2 ± 2.77	30.4 ± 4.50	59.8 ± 7.20	21.7 ± 0.99	10.8 ± 0.62	NA
ADF	39.9 ± 1.52	32.6 ± 1.30	38.2 ± 1.88	16.1 ± 0.64	43.2 ± 5.38	15.0 ± 1.86	3.93 ± 0.99	NA
Acid detergent lignin	5.90 ± 0.47	6.98 ± 0.93	5.28 ± 0.42	6.15 ± 0.40	1.90 ± 0.69	4.53 ± 0.30	1.00 ± 0.34	NA
Ether extract	2.43 ± 0.41	4.73 ± 0.57	4.53 ± 0.53	2.20 ± 0.26	2.83 ± 1.15	19.7 ± 1.06	4.55 ± 1.42	1.20 ± 0.24
Ash	5.83 ± 0.45	9.44 ± 0.67	8.64 ± 1.59	6.09 ± 0.10	4.67 ± 0.34	5.90 ± 0.30	2.10 ± 0.44	10.2 ± 0.10
WSC[4]	12.3	4.65 ± 1.32	5.75 ± 1.21	4.30	2.60	8.40	1.90	67.3
Fructose	0.59	1.27 ± 0.18	1.32 ± 0.50	0.10	0.10	0.23	0.13	5.20 ± 2.44
Glucose	0.42	0.71 ± 0.12	0.90 ± 0.80	0.34	0.56	0.23	0.21	3.71 ± 1.07
Sucrose	1.19	1.90 ± 0.25	1.23 ± 0.11	2.87	1.02	5.48	1.28	31.8 ± 5.64
Maltose	ND[5]	ND	ND	ND	ND	ND	ND	0.31 ± 0.09
Raffinose	ND	ND	ND	2.22	0.46	1.09	ND	ND
Stachyose	ND	ND	ND	0.25	0.83	4.12	ND	0.08 ± 0.14
Mannitol	1.22	0.54 ± 0.20	0.74 ± 0.53	0.07	0.03	ND	0.07	0.10 ± 0.03
Total individual sugars[6]	3.42	4.42 ± 0.51	4.19 ± 0.76	5.85	3.00	11.2	1.69	41.2 ± 2.09
Starch	0.78 ± 0.10	2.18 ± 0.62	0.58 ± 0.17	2.85 ± 1.64	2.60 ± 1.57	5.60 ± 1.25	67.1 ± 4.05	NA
NFC	16.0 ± 0.67	21.2 ± 1.79	12.8 ± 1.01	22.9 ± 5.98	18.9 ± 6.05	14.5 ± 2.58	73.8 ± 2.48	NA
SDG[7]	NA	NA	NA	1.80 ± 0.13	NA	NA	NA	NA
Ca	0.40 ± 0.03	1.06 ± 0.05	0.58 ± 0.18	0.37 ± 0.03	0.49 ± 0.05	0.26 ± 0.03	0.06 ± 0.02	0.35 ± 0.31
P	0.20 ± 0.02	0.35 ± 0.01	0.31 ± 0.03	0.81 ± 0.02	0.16 ± 0.03	0.55 ± 0.03	0.29 ± 0.01	0.06 ± 0.01
Mg	0.24 ± 0.03	0.34 ± 0.02	0.21 ± 0.04	0.56 ± 0.01	0.24 ± 0.01	0.23 ± 0.01	0.12 ± 0.02	0.41 ± 0.02
K	1.36 ± 0.22	2.38 ± 0.23	2.42 ± 0.08	1.18 ± 0.02	1.08 ± 0.06	1.57 ± 0.06	0.48 ± 0.09	3.75 ± 0.34
S	0.21 ± 0.01	0.27 ± 0.03	0.23 ± 0.01	0.38 ± 0.01	0.14 ± 0.02	0.32 ± 0.01	0.12 ± 0.02	0.24 ± 0.00
Na	0.07 ± 0.01	0.06 ± 0.01	0.09 ± 0.01	0.09 ± 0.04	0.01 ± 0.00	0.03 ± 0.03	0.02 ± 0.02	0.09 ± 0.01
Fe, mg/kg	130 ± 77.6	238 ± 16.0	143 ± 53.5	166 ± 26.9	568 ± 131	328 ± 77.0	61.3 ± 22.2	349 ± 14.1
Cu, mg/kg	6.50 ± 0.58	11.7 ± 0.58	8.33 ± 0.58	19.5 ± 0.58	7.00 ± 1.15	11.3 ± 0.50	3.50 ± 1.29	32.3 ± 0.58
Mn, mg/kg	54.3 ± 9.18	27.0 ± 2.65	43.3 ± 1.53	49.5 ± 5.69	19.8 ± 1.71	31.3 ± 1.53	10.0 ± 4.08	13.3 ± 0.58

Zn, mg/kg	26.8 ± 3.10	26.3 ± 2.08	26.3 ± 3.21	70.5 ± 3.87	49.3 ± 4.11	48.8 ± 9.22	28.0 ± 6.78	16.3 ± 0.58
Mo, mg/kg	1.53 ± 0.15	2.07 ± 0.06	1.73 ± 0.75	1.08 ± 0.21	0.40 ± 0.08	1.03 ± 0.10	0.34 ± 0.40	1.67 ± 0.21

[1]Samples of grass hay, flaxseed meal, soyhulls, roasted soybean, and ground corn were pooled across periods before analyzing for water-soluble carbohydrates and individual sugars; samples of liquid molasses were pooled across periods before analyzing for water-soluble carbohydrates.
[2]Sugarcane liquid molasses (Buffalo Molasses, LCC; North Java, NY).
[3]NA = not analyzed.
[4]WSC = water-soluble carbohydrates; analyzed colorimetrically (Hall et al., 1999).
[5]ND = not detected.
[6]Total individual sugars analyzed chromatographically by HPLC (Analab, Fulton, IL).
[7]SDG = secoisolariciresinol diglucoside.

Table 5. Ingredient and nutritional composition (% of DM, unless otherwise noted) of the experimental diets

	Dietary levels of liquid molasses			
Item	0%	4%	8%	12%
Ingredient composition				
Mixed mostly grass baleage	31.2	31.2	31.2	31.2
Mixed mostly legume baleage	20.8	20.8	20.8	20.8
Grass hay	8.00	8.00	8.00	8.0
Sugarcane liquid molasses	0.00	4.00	8.00	12.0
Ground corn	12.0	8.00	4.00	0.00
Flaxseed meal	15.0	15.0	15.0	15.0
Roasted soybean	2.50	2.50	2.50	2.50
Soyhulls	8.50	8.50	8.50	8.50
Minerals-vitamins premix[1]	2.00	2.00	2.00	2.00
Nutritional composition[2]				
DM, % of fresh matter	52.8	50.5	50.7	50.1
CP	19.0	18.8	18.8	18.6
NDF	44.2	43.7	43.3	42.9
ADF	28.8	28.7	28.5	28.3
Acid detergent lignin	4.89	4.85	4.81	4.77
Starch	9.53	6.85	4.16	1.48
NFC	25.8	26.2	26.7	27.0
Total individual sugars added[3]	0.00	1.65	3.30	4.95
Total individual sugars[4]	3.90	5.48	7.05	8.63
WSC added[5]	0.00	2.69	5.38	8.08
Total WSC[6]	4.02	5.21	9.74	12.9
Ash	6.84	7.16	7.28	7.81
Ether extract	4.20	4.06	3.93	3.79
Ca	0.94	0.96	0.97	0.99
P	0.39	0.36	0.37	0.34
K	1.72	1.85	1.98	2.12
Na	0.24	0.25	0.25	0.25
NE_L,[7] Mcal/kg of DM	1.57	1.56	1.56	1.56
Fatty acids,[8] g/kg of DM				
16:0	3.83	3.84	3.48	3.44
18:0	0.74	0.76	0.68	0.69
cis-9 18:1	3.10	2.93	2.49	2.42
*c*is-9, *cis*-12 18:2	6.59	6.40	5.66	5.28
cis-9, *cis*-12, *cis*-15 18:3	6.72	6.76	6.69	6.78
Others and unidentified	1.62	1.73	1.95	2.09
Total	22.6	22.4	20.9	20.7

[1]The minerals and vitamins premix contained (DM basis): 19.9% Ca, 1.11% P, 9.50% Mg, 0.11% K, 9.19% Na, 14.1% Cl, 13.6 mg/kg Se, 319 mg/kg Cu, 2,097 mg/kg Mn, 2,337 mg/kg Zn, 1,624 mg/kg Fe, 31.2 mg/kg I, 96,068 IU/kg vitamin A, 22,130 IU/kg vitamin D_3, and 513 IU/kg vitamin E.

[2]The nutritional composition of the diets was calculated based on the individual nutritional composition of the dietary ingredients as reported in Table 1.
[3]Total individual sugars added from liquid molasses analyzed chromatographically by HPLC (Analab, Fulton, IL).
[4]Dietary total individual sugars (liquid molasses plus remaining feedstuffs) analyzed chromatographically by HPLC (Analab, Fulton, IL).
[5]Water-soluble carbohydrates (WSC) added from liquid molasses analyzed colorimetrically (Hall et al., 1999).
[6]Total WSC (liquid molasses plus remaining feedstuffs) analyzed colorimetrically (Hall et al., 1999).
[7]Determined using the NRC (2001) including actual feed nutritional composition and animal variables (i.e., DMI, milk yield and composition, DIM, and BW).
[8]Dietary fatty acid profile determined using TMR samples.

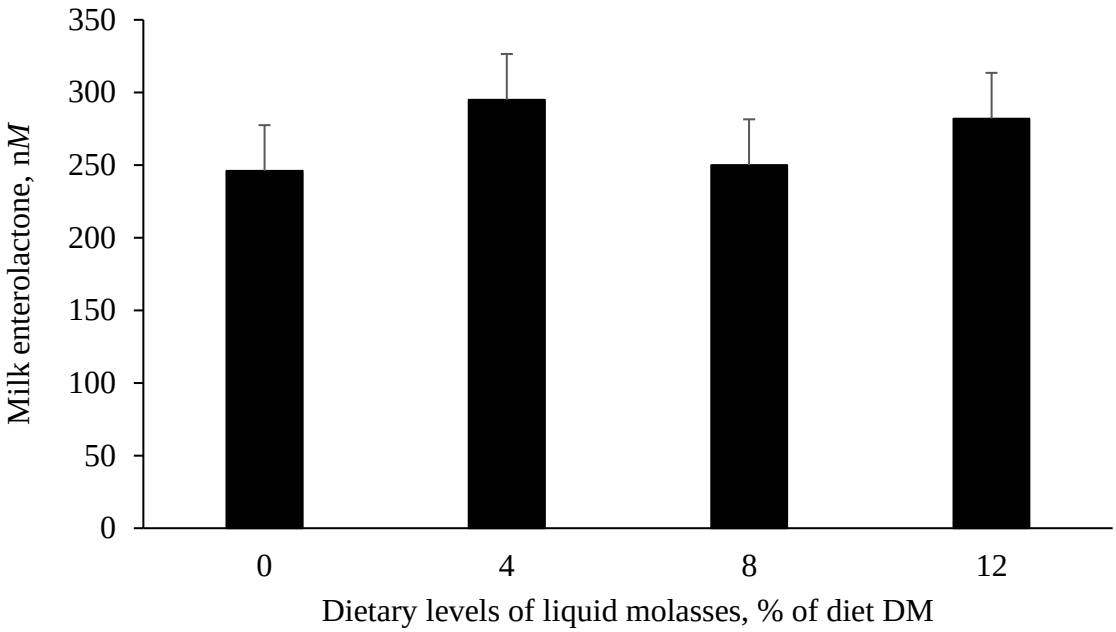

Figure 5. Effects of replacing ground corn with incremental amounts of sugarcane liquid molasses on milk concentration of enterolactone (n*M*) in Jersey cows fed flaxseed meal and low-starch diets (12:0, 8:4, 4:8, and 0:12 ground corn:liquid molasses dietary ratios). Data are presented as least square means ± SEM. Orthogonal polynomials were used to test linear, quadratic, and cubic effects; significance was declared at $P \leq 0.05$ and trends at $0.05 < P \leq 0.10$. A trend ($P = 0.08$) for cubic effect was observed.

Table 6. Effects of replacing ground corn with incremental amounts of sugarcane liquid molasses on production, milk composition, feed and milk N efficiencies, plasma concentrations of antioxidant enzymes, and BW change in Jersey cows fed flaxseed meal and low-starch diets

	Dietary levels of liquid molasses[1]					Contrasts (*P*-value)[2]		
Item	0%	4%	8%	12%	SEM	Linear	Quadratic	Cubic
Plasma SOD,[3] U/mL	1.81	1.90	1.70	1.85	0.10	0.83	0.71	0.06
Plasma GPx,[4] nmol/min per mL	120	116	123	117	9.81	0.88	0.91	0.39
Plasma catalase, nmol/min per mL	24.9	29.2	28.6	20.5	4.69	0.49	0.14	0.90
SDG intake,[5] g/d	51.4	50.2	48.5	47.6	1.25	<0.01	0.86	0.77
K intake, g/d	330	345	358	370	9.55	<0.001	0.84	0.98
Na intake, g/d	45.8	46.5	45.1	44.8	1.27	0.30	0.61	0.46
DMI, kg/d	19.1	18.6	18.0	17.6	0.48	<0.01	0.95	0.90
N intake, g/d	595	572	552	530	14.2	<0.001	0.92	0.92
Milk, kg/d	18.9	17.9	17.8	16.8	0.55	<0.001	0.98	0.22
4% FCM,[6] kg/d	22.9	21.5	21.7	19.6	0.77	<0.001	0.56	0.12
ECM,[7] kg/d	24.9	23.7	23.6	21.4	0.79	<0.001	0.57	0.12
Milk/DMI, kg/kg	1.00	0.97	0.99	0.97	0.03	0.50	0.71	0.39
4% FCM/DMI, kg/kg	1.21	1.16	1.21	1.13	0.04	0.26	0.80	0.14
ECM/DMI, kg/kg	1.32	1.26	1.31	1.23	0.04	0.25	0.81	0.14
Milk N/N intake, %	19.9	19.7	20.2	19.7	0.45	0.99	0.89	0.41
Milk fat, %	5.29	5.37	5.29	5.25	0.15	0.48	0.40	0.56
Milk fat, kg/d	0.99	0.96	0.94	0.87	0.04	<0.01	0.25	0.48
Milk true protein, %	4.03	4.05	4.02	3.99	0.09	0.24	0.38	0.74
Milk true protein, kg/d	0.75	0.72	0.71	0.66	0.02	<0.001	0.59	0.28
Milk lactose, %	4.66	4.65	4.64	4.60	0.02	<0.01	0.29	0.60
Milk lactose, kg/d	0.88	0.84	0.83	0.77	0.03	<0.001	0.85	0.18
MUN, mg/dL	16.8	16.3	15.9	16.1	0.48	0.02	0.22	0.59
Milk SCC, 1,000 cells/mL	174	188	168	155	67.8	0.24	0.37	0.56
PUN, mg/dL	26.0	24.7	24.7	24.3	0.73	0.07	0.42	0.52
BW, kg	464	462	467	468	8.30	0.03	0.44	0.14
BW change, kg/d	-0.01	0.05	0.08	0.11	0.057	0.13	0.79	0.89

[1]Ground corn:liquid molasses dietary ratios: 12:0, 8:4, 4:8, and 0:12.

[2]Orthogonal polynomials were used to test linear, quadratic, and cubic effects; significance was declared at $P \leq 0.05$ and trends at $0.05 < P \leq 0.10$.
[3]SOD = superoxide dismutase.
[4]GPx = glutathione peroxidase.
[5]SDG = secoisolariciresinol diglucoside.
[6]4% FCM = [0.40 × milk yield (kg/d)] + [15 × milk fat yield (kg/d)] (Gaines and Davidson, 1923).
[7]ECM = [0.327 × milk yield (kg/d)] + [12.95 × milk fat yield (kg/d)] + [7.2 × milk true protein yield (kg/d)] (Orth, 1992).

Table 7. Effects of replacing ground corn with incremental amounts of sugarcane liquid molasses on apparent total-tract digestibility of nutrients and urinary excretion of nitrogenous metabolites in Jersey cows fed flaxseed meal and low-starch diets

	Dietary levels of liquid molasses[1]					Contrasts (*P*-value)[2]		
Item	0%	4%	8%	12%	SEM	Linear	Quadratic	Cubic
Apparent total-tract digestibility								
DM, % of DMI	71.5	70.0	71.0	70.9	0.66	0.74	0.28	0.20
OM, % of OM intake	72.7	71.2	72.1	72.1	0.64	0.71	0.18	0.22
NDF, % of NDF intake	64.7	64.1	65.0	65.2	0.93	0.56	0.64	0.59
ADF, % of ADF intake	67.1	66.5	67.3	66.8	0.99	0.98	0.97	0.55
CP, % of CP intake	73.9	71.4	72.0	71.1	0.72	0.01	0.24	0.14
Starch, % of starch intake	99.98	99.97	99.95	99.90	0.01	<0.001	<0.001	0.64
Urinary volume and excretion								
Urine, L/d	24.3	24.9	26.9	25.8	0.79	0.02	0.16	0.08
Creatinine, m*M*	4.19	4.10	3.84	3.88	0.11	<0.01	0.45	0.24
Uric acid, mmol/d	36.9	40.1	35.4	37.2	2.55	0.63	0.73	0.08
Allantoin, mmol/d	154	172	168	163	12.1	0.65	0.30	0.70
Total PD,[3] mmol/d	191	212	204	200	13.1	0.71	0.31	0.51
PD:Creatinine ratio[4]	1.93	2.14	2.05	2.15	0.13	0.33	0.68	0.41
Total N, g/d	244	234	233	213	9.14	0.02	0.59	0.55
Total N, % of N intake	41.5	41.7	42.5	40.5	1.97	0.78	0.59	0.70
Urea N, g/d	125	127	120	113	7.06	0.17	0.58	0.75
Urea N, % total urinary N	51.9	55.5	53.5	53.6	2.43	0.75	0.44	0.44
Urea N, % of N intake	21.4	22.6	22.0	21.7	1.54	0.97	0.61	0.74

[1]Ground corn:liquid molasses dietary ratios: 12:0, 8:4, 4:8, and 0:12.

[2]Orthogonal polynomials were used to test linear, quadratic, and cubic effects; significance was declared at $P \leq 0.05$ and trends at $0.05 < P \leq 0.10$.

[3]Total purine derivatives (PD) = uric acid + allantoin.

[4]PD:Creatinine ratio = urinary excretion of total PD (mmold/d) ÷ urinary excretion of creatinine (mmol/d).

Table 8. Effects of replacing ground corn with incremental amounts of sugarcane liquid molasses on the milk proportions of SFA and UFA in Jersey cows fed flaxseed meal and low-starch diets

	Dietary levels of liquid molasses[1]					Contrasts (*P*-value)[2]		
Fatty acids (FA), g/100 g	0%	4%	8%	12%	SEM	Linear	Quadratic	Cubic
4:0	4.08	4.07	4.15	4.10	0.10	0.26	0.55	0.07
6:0	2.32	2.31	2.33	2.29	0.03	0.28	0.20	0.08
8:0	1.32	1.32	1.32	1.29	0.02	<0.01	0.28	0.39
10:0	3.28	3.26	3.22	3.15	0.08	<0.01	0.52	0.99
11:0	0.065	0.065	0.062	0.065	0.0053	0.77	0.38	0.26
12:0	3.97	3.95	3.87	3.81	0.13	0.01	0.71	0.67
13:0	0.10	0.10	0.10	0.11	0.006	0.60	0.26	0.51
14:0	12.3	12.2	12.3	12.2	0.16	0.52	0.61	0.41
iso 14:0	0.14	0.14	0.13	0.13	0.004	<0.01	0.09	0.28
cis-9 14:1	0.88	0.90	0.90	0.91	0.026	0.24	0.74	0.57
15:0	1.17	1.17	1.20	1.26	0.03	<0.001	0.01	0.64
iso 15:0	0.35	0.36	0.36	0.37	0.008	0.01	0.86	0.32
anteiso 15:0	0.49	0.49	0.49	0.50	0.012	0.43	0.11	0.30
16:0	33.4	34.1	34.7	35.2	0.54	<0.001	0.79	0.97
iso 16:0	0.27	0.26	0.24	0.24	0.008	<0.001	0.28	0.32
cis-9 16:1	1.03	1.08	1.10	1.14	0.05	<0.001	0.53	0.59
17:0	0.55	0.57	0.58	0.61	0.006	<0.001	0.07	0.31
iso 17:0	0.31	0.32	0.32	0.33	0.006	0.01	0.20	0.76
anteiso 17:0	0.37	0.38	0.37	0.38	0.008	0.15	0.30	0.13
cis-9 17:1	0.14	0.15	0.16	0.17	0.005	<0.001	0.76	0.34
18:0	11.1	10.7	10.3	9.94	0.23	<0.001	0.71	0.88
cis-9 18:1	13.6	13.1	13.1	12.9	0.26	<0.001	0.71	0.83
cis-11 18:1	0.28	0.28	0.28	0.28	0.008	0.75	0.87	0.60
cis-12 18:1	0.15	0.15	0.14	0.13	0.005	<0.001	0.35	0.63
trans 6-8 18:1	0.19	0.18	0.18	0.17	0.004	<0.001	0.78	0.57
trans-9 18:1	0.14	0.14	0.13	0.13	0.003	<0.001	0.85	0.61
trans-10 18:1	0.19	0.19	0.19	0.18	0.005	0.08	0.15	0.50
trans-11 18:1	1.51	1.50	1.51	1.56	0.05	0.15	0.30	0.95
trans-12 18:1	0.27	0.26	0.25	0.23	0.005	<0.001	0.83	0.92

trans-15 18:1	0.21	0.20	0.19	0.19	0.005	<0.001	0.72	0.92
cis-9, *cis*-12 18:2	1.36	1.37	1.31	1.25	0.04	<0.001	0.07	0.38
cis-9, *trans*-11 18:2	0.54	0.56	0.56	0.60	0.017	<0.001	0.36	0.33
cis-9, *cis*-12, *cis*-15 18:3	0.61	0.62	0.65	0.66	0.021	<0.01	0.74	0.62
20:0	0.17	0.16	0.16	0.16	0.003	<0.01	0.30	0.77
cis-9 20:1	0.083	0.081	0.080	0.081	0.0048	0.73	0.68	0.98
cis-8, *cis*-11, *cis*-14 20:3	0.078	0.080	0.076	0.076	0.0042	0.18	0.37	0.16
cis-5, *cis*-8, *cis*-11, *cis*-14 20:4	0.071	0.073	0.072	0.074	0.0031	0.24	0.73	0.37
22:0	0.072	0.071	0.071	0.075	0.0020	0.17	0.07	0.83
Unidentified FA	2.75	2.79	2.85	2.98	0.07	<0.001	0.35	0.73
Sum and ratio of FA								
Σ odd-chain[3]	2.04	2.06	2.09	2.20	0.05	<0.001	0.02	0.40
Σ branched-chain[4]	1.94	1.95	1.91	1.95	0.04	0.96	0.45	0.23
Σ < 16-C[5]	28.1	28.0	28.1	27.7	0.30	0.11	0.45	0.49
Σ 16-C[6]	34.4	35.2	35.8	36.3	0.55	<0.001	0.75	0.93
Σ 18-C[7]	19.0	18.8	18.5	18.3	0.33	<0.01	0.84	0.76
Σ *cis*-18:1	14.1	13.8	13.5	13.3	0.27	<0.001	0.75	0.81
Σ *trans*-18:1	2.50	2.46	2.44	2.45	0.06	0.40	0.63	0.93
trans-11/*trans*-10 ratio	8.15	7.92	8.36	8.95	0.21	<0.001	0.01	0.47
Δ^9-desaturase index$_{cis\text{-}9,\ trans\text{-}11\ 18:2}$[8]	26.5	27.1	27.2	27.7	0.68	<0.01	0.74	0.48

[1]Ground corn:liquid molasses dietary ratios: 12:0, 8:4, 4:8, and 0:12.

[2]Orthogonal polynomials were used to test linear, quadratic, and cubic effects; significance was declared at $P \leq 0.05$ and trends at $0.05 < P \leq 0.10$.

[3]Σ odd-chain FA = 11:0 + 13:0 + 15:0 + 17:0 + *cis*-9 17:1.

[4]Σ branched-chain FA = *iso* 14:0 + *iso* 15:0 + *anteiso* 15:0 + *iso* 16:0 + *iso* 17:0 + *anteiso* 17:0.

[5]Σ <16-C FA (originate from de novo synthesis in the mammary gland) = 4:0 + 6:0 + 8:0 + 10:0 + 12:0 + 14:0 + *cis*-9 14:1.

[6]Σ 16-C FA (originate from de novo synthesis in the mammary gland and extraction from plasma) = 16:0 + *cis*-9 16:1.

[7]Σ 18-C FA (originate from plasma extraction) = 18:0 + all 18-C UFA.

[8]Δ^9-desaturase index$_{cis\text{-}9,\ trans\text{-}11\ 18:2}$ = *cis*-9, *trans*-11 18:2 ÷ (*trans*-11 18:1 + *cis*-9, *trans*-11 18:2) × 100.

CHAPTER III: EFFECTS OF FEEDING SUCROSE AND FLAXSEED OIL ON MILK ENTEROLACTONE CONCENTRATION AND PERFORMANCE IN HOLSTEIN COWS FED FLAXSEED MEAL

INTRODUCTION

The mammalian lignans, enterolactone (**EL**) and enterodiol (**ED**) are polyphenolic compounds synthesized from plant lignans. Enterolactone and ED have a wide range of biological activities, including estrogen-like, antiestrogenic, antioxidant, anti-inflammatory, anticarcinogenic, and cardioprotective effects (Adolphe et al., 2010; Högger, 2013; Imran et al., 2015). Flaxseed (*Linum usitatissimum* L.) is the richest source of the lignan secoisolariciresinol diglycoside (**SDG**), which is a precursor for the synthesis of mammalian lignans by the gut microbiota of humans (Thompson et al., 1991; Gaya et al., 2016) and ruminants (Gagnon et al., 2009; Zhou et al., 2009). Feeding incremental amounts of flaxseed meal (**FM**) to dairy cows linearly increased the concentration of EL in milk (Petit and Gagnon, 2009). Enterolactone-enriched milk can be used as a source of EL for humans not only because milk is consumed by a large part of the world population, but also due to poor and variable intake of plant lignans worldwide, including in the United States (de Kleijn et al., 2001).

In ruminants, plant lignans are metabolized to mammalian lignans primarly through the action of ruminal microbes (Gagnon et al., 2009; Zhou et al., 2009). Despite the importance of the ruminal microbiota on lignans metabolism in dairy cows, just a couple of studies have investigated lignans metabolism in the rumen and how dietary changes could impact this process (Gagnon et al., 2009; Zhou et al., 2009). *Prevotella spp*. has been identified as the major ruminal microbe involved into the process of converting plant lignans to mammalian lignans in dairy cows (Schogor et al., 2014). Therefore, changes in the diet that favor the growth of *Prevotella spp.* in the rumen could potentially improve the conversion of plant lignans to mammalian lignans ultimately increasing the concentration of EL in milk. A study carried out by Li et al. (2015) showed that when steers were fed diets containing 4% of flaxseed oil (**FO**) the genus

Prevotella dominated the ruminal bacterial community. Additionally, Brito et al. (2015) reported that dairy cows fed liquid molasses (**LM**) and FM had greater concentration of milk EL than those fed ground corn and FM. This result suggests that sucrose may select for ruminal microorganisms with greater capacity to convert SDG to EL compared with starch. Despite these findings, whether feeding sucrose or FO would change the ruminal microbiota population resulting in increased SDG conversion to EL in cows fed FM has yet to be elucidated.

We hypothesized that cows fed FM-based diets supplemented with sucrose and FO would produce milk with greater concentration of EL than those fed soybean meal (**SBM**) and ground corn. We also hypothesized that sucrose and FO may synergistically interact to increase the output of ruminal EL by improving SDG metabolism into the rumen, leading to improved EL concentration in milk. The objective of this study was to evaluate the effects of feeding sucrose and FO, alone or in association in FM-based diets on production, milk composition, milk EL concentration, plasma concentrations of thiobarbituric acid reactive substances (**TBARS**) and urea N (**PUN**), and apparent total-tract digestibility of nutrients in Holstein cows.

MATERIALS AND METHODS

Experiment date and location

The 100-d long experiment was conducted at the University of New Hampshire Fairchild Dairy Teaching and Research Center (Durham, NH) from November 3rd, 2016 to February 12th, 2017. Care and handling of the animals used in the study were conducted as outlined in the guidelines of the University of New Hampshire Institutional Animal Care and Use Committee (protocol no. 160603).

Animals, Experimental Design, and Diets

Sixteen Holstein cows (4 ruminally-cannulated) were used. Eight cows were multiparous cows averaging (mean ± SD) 104 ± 24 days in milk (**DIM**), 743 ± 29.8 kg of body weight (**BW**), and 43.1 ± 16.5 kg/d of milk at the beginning of the study. The remaining 8 cows were primiparous cows averaging 85 ± 42 DIM, 611 ± 32.1 kg of BW, and 34.6 ± 5.10 kg/d of milk at the beginning of the study. Cows were randomly assigned to treatment sequences in a replicated 4 × 4 Latin square design. Within each square, treatment sequences were balanced for potential carryover effects in subsequent periods as each treatment immediately preceded and followed every other exactly once (Williams, 1949; Kim and Stein, 2009). Animals were distributed in balanced squares resulting in 2 squares of multiparous cows (square1 = 84 ± 15 DIM, 736 ± 38.0 kg of BW, and 41.4 ± 10.9 kg/d of milk at the beginning of the experiment; square 2 = 125 ± 10 DIM, 750 ± 14.8 kg of BW, and 45.2 ± 19.9 kg/d of milk at the beginning of the experiment) and two squares of primiparous cows (square 3 = 120 ± 24 DIM, 623 ± 21.2 kg of BW, and 37.3 ± 4.16 kg/d of milk at the beginning of the experiment; square 4 = 47 ± 18 DIM, 598 ± 35.7 kg of BW, and 31.0 ± 13.8 kg/d of milk at the beginning of the experiment).

Each experimental period lasted 25 d with 18 d for diet adaptation and 7 d for data and sample collection. Cows were fed diets formulated to meet or exceed the nutritional requirements of Holstein cow producing 40 kg/d of milk with 3.20% milk protein and 3.5% milk fat using NRC (2001) ration-evaluation software. Treatments were fed (DM basis) as total mixed ration (**TMR**) consisting of a 60:40 forage-to-concentrate ratio and included: a negative control diet (**-CTRL**; 8% SBM plus 23% ground corn); 15% FM +10.7% ground corn + 5% sucrose (**SUCR**); 15% FM + 15.4% ground corn + 3% FO (**OIL**); and 15% FM +10.2% ground corn + 5% sucrose + 3% FO (**COMBO**).

Animal Feeding and Feed Sampling and Analyses

Cows were housed in a tie-stall barn equipped with individual feed tubs and had free access to water throughout the experiment. Diets were mixed twice a day using a vertical TMR mixer (Super Data Ranger; American Calan Inc., Northwood, NH) and individually offered to the cows at 0530 and 1530 h. Feed intake was individualized using wooden feed tubs (90 x 90 x 90 cm) for each cow. In order to account for difference in feeding interval approximately 40% of the total daily ration was fed in the morning and the remaining 60% in the afternoon feeding. The amount of TMR offered to cows was adjusted daily to allow refusals of 5 to 10% of intake. Refusals were collected and weighed daily prior the afternoon feeding using the Super Data Ranger. Feed intake was recorded individually by subtracting the amount of TMR offered daily from the amount of refusals. Samples of TMR, feeds, and refusals (composited by diet) were collected daily during each sampling week and composited by period. All feed samples were dried in a forced-air oven (55°C, 48 h), ground to pass through a 1-mm screen (Wiley mill; Arthur H. Thomas, Philadelphia, PA), and shipped to a commercial laboratory (Dairy One Cooperative Inc., Ithaca, NY) for nutrient analyses. The following analyses were performed in feed samples: dry matter (**DM**) (method 930.15; AOAC International, 2006), total N (method 990.03; AOAC International, 2006), neutral detergent fiber (**NDF**) (method 6; Ankom Technology, Fairport, NY; solutions as in Van Soest et al., 1991), acid detergent fiber (**ADF**) (method 5; Ankom Technology; solutions as in method 973.18; AOAC International, 1998), crude fat (method 2003.05; AOAC International, 2006), ash (method 942.05; AOAC International, 2006), and acid detergent lignin [method 9 in a ANKOM Daisy Incubator; solutions as in method 973.18 (AOAC International, 2016)]. Individual minerals (Ca, P, Mg, K, Na, Fe, Zn, Cu, Mn, Mo, S, and Se) were analyzed (Dairy One Cooperative Inc.) in all feed

samples using an iCAP 6300 Intrepid Inductively Coupled Plasma Radial Spectrometer (Thermo Fisher Scientific Inc., Waltham, MA) after microwave digestion (CEM application note for acid digestion; CEM, Matthews, NC). Refusals samples were analyzed for DM, ash, NDF, ADF, and total N according to methods and procedures used for the feed samples described above.

Animal Performance and Milk Sampling and Analyses

Body weights were measured during 3 consecutive days before the beginning of the study and during the last 3 d of each sampling period to determine BW change. Cows were milked twice daily at approximately 0500 and 1530 h, and milk yield was recorded at each milking throughout the experiment. Milk samples were collected during 4 consecutive milkings (d 23 and 24) for each sampling period, stored in tubes containing 2-bromo-2-nitropropan-1,3 diol, pooled by cow according to morning and evening milk weights, and refrigerated at 4°C until shipped to Dairy One Cooperative Inc. for determination of fat, true protein, lactose, MUN and SCC by mid-infrared reflectance spectroscopy. Subsamples of milk without preservative were collected concurrently, pooled using the same procedure described above, and stored at -20°C until analyzed for EL. Milk EL was extracted and hydrolyzed [β-glucuronidase/arylsulfatase from *Helix pomatia* (Roche-Diagnostics; Laval, QC, Canada)] according to procedures described previously (Gagnon et al., 2009). After extraction and hydrolysis, EL was analyzed colorimetrically in quadruplicate using a competitive commercial enzymatic immunoassay (assay kit no. 500520; Cayman Chemical Co., Ann Arbor, MI) that recognizes both enantiomeric forms of EL with a UV/visible spectrophotometer (Beckman Coulter Inc., Brea, CA) set at a wavelength of 405 nm.

Blood sampling and Analyses

Blood samples were collected from the coccygeal vein or artery approximately 4 h after the morning feeding on the last day of each experimental period using 10-mL vacutainer tubes containing K_3-EDTA (Covidien, Minneapolis, MN). After collection, tubes were kept on ice until processing. Samples were centrifuged (3,300 × *g*, 20 min, 4°C) and 2 aliquots of plasma were collected. The first aliquot was stored at -80°C for determination of TBRAS, while the second aliquot was stored at -20°C for determination of PUN. After thawed at room temperature, plasma samples were analyzed colorimetrically with a UV/Vis spectrophotometer (Beckman Coulter Inc.) for TBARS (assay kit # 706002; Cayman Chemical Co., Ann Arbor, MI) and PUN (diacetyl-monoxime method; Rosenthal, 1955) set at wavelengths of 530 and 540 nm, respectively.

Fecal and Urinary Sampling and Analyses

Fecal and urinary samples were collected once daily for 3 consecutive d (d 23 to 25 d) at 0700 h, 1200 h, and 1800 h. Fecal grab samples were collected by stimulating defecation or directly from the rectum. After collection, fecal samples were stored at -20°C in plastic bags and later dried in a forced-air oven at 55°C for approximately 72 h. Following oven-drying, samples were ground to pass through a 1-mm screen (Wiley mill) and pooled by cow based on dry weight over the 3 d to obtain a single composite sample per cow per period. Fecal samples were analyzed for DM, ash, total N, NDF, ADF, as described previously. Approximately 0.5 g of feces, feeds, and TMR samples were weighed into Ankom F57 bags (Ankom Technology), placed in 1 larger laundry bag, and inserted in the rumen of 1 ruminally-cannulated lactating Holstein cow for 12 d. After removal from the rumen, bags were rinsed with tap water and

analyzed for ADF as described above. Indigestible ADF was used as an intrinsic marker to estimate fecal output of DM and apparent total-tract digestibility of nutrients (Cochran et al., 1986; Huhtanen et al., 1994).

Spot samples of urine were collected concurrently with fecal samples by stimulation of the pudendal nerve massaging the area bellow the vulva. After collection, urinary samples were immediately transported to the laboratory for processing. Subsamples of urine from each cow (approximately 2.7 mL/time point) were pooled over 3 d into 50-mL centrifuge tubes containing 32 mL of 0.072 *N* H_2SO_4 and stored at -20°C until analyses. Urine samples were thawed at room temperature and analyzed colorimetrically for creatinine (assay kit no. 500701; Cayman Chemical Co.), allantoin (Chen et al., 1992), uric acid (assay kit no. 1045-225; Stanbio Laboratory, Boerne, TX), and total N (microKjeldahl analysis; AOAC, 2016; Dairy One Cooperative Inc.). Allantoin and uric acid were read at wavelengths of 522 and 520 nm, respectively, on a UV/visible spectrophotometer (Beckman Coulter Inc.). Daily urinary volume was estimated from the urinary concentration of creatinine assuming a constant creatinine excretion rate of 29 mg/kg of BW (Valadares et al., 1999). Urinary excretion of allantoin, uric acid, total purine derivatives (**PD** = allantoin + uric acid), total N, and urea N were calculated by multiplying the urinary volume by the concentration of each metabolite in urine.

Statistical Analysis

Data were analyzed using the MIXED procedure of SAS (SAS version 9.4; SAS Inst. Inc., Cary, NC) according to a replicated 4 × 4 Latin square design. The following model was used for all variables:

$$Y_{ijkl} = \mu + S_i + P_j + C_{k(i)} + T_l + S \times T_{il} + E_{ijkl},$$

where Y_{ijkl} = dependent variable, μ = overall mean, S_i = fixed effect of ith square, P_j = fixed effect of jth period, $C_{k(i)}$ = random effect of kth cow within ith square, T_l = fixed effect of lth treatment, $S \times T_{il}$ = interaction between ith square and lth treatment, and E_{ijkl} = error term. All reported values are least squares means and standard error of the mean. The Tukey test was performed to test for significant differences among treatments. Significance was declared at $P \leq 0.05$ and trends at $0.05 < P \leq 0.10$. Milk concentration of EL was transformed (natural log) before statistical analyses, but results presented herein were reported as adjusted mean values of quadruplicate runs on the original scale of measurements as done previously (Petit and Gagnon, 2009; Brito et al., 2015).

RESULTS AND DISCUSION

Feeds and Diets Nutritional Composition

The nutritional composition of the feedstuffs used, as well as ingredient and nutritional composition of the experimental diets are presented in Tables 9 and 10, respectively. The concentration of SDG in FM averaged 1.30% (DM basis) and was lower than the concentrations reported by Brito et al. (2015) (mean = 1.66%). Flaxseed meal had lower concentration of CP than SBM (37 and 47%, respectively), and much higher concentration of NDF (36.3 and 14.10 %, respectively). Dietary concentrations of CP (mean = 17.2 %), NDF (mean = 32.1%), ADF (mean = 18.2%), and NE_L (mean = 1.59 Mcal/kg of DM) were similar across experimental diets (Table 10). However, the concentration of non-fiber carbohydrates (**NFC**) was slightly greater in the –CRTL diet compared to those containing FM, especially the OIL diet (Table 10). The -CTRL diet had greater amount of ground corn compared with FM diets, which explains the increased concentration of NFC.

Production and Nutrient Digestibility

Dry matter intake, milk yield, and milk composition are presented in Table 11. Cows fed FM-based diets (i.e., SUCR, OIL, or COMBO) had lower DMI ($P < 0.001$) compared with those fed the –CRTL diet. Lowered DMI can be in part explained by decreased apparent total-tract digestibilities of DM ($P = 0.01$), OM ($P < 0.01$), and ADF ($P = 0.05$) in cows fed FM vs. soybean meal (-CRTL) diets. Within cows fed FM, the reduction in DMI was greatest in cows fed the OIL diet with no difference between SUCR and COMBO treatments. The effect of FO supplementation on DMI of dairy cows has been inconsistent in the literature. Chilliard et al. (2009) reported no effect on DMI in cows fed diets based on corn silage and grass hay and supplemented with oil from whole crude linseed at 4.2% of dietary DM. Similarly, Benchaar et al. (2012) reported no effect on DMI in dairy cows fed incremental amounts (0, 2, 3, or 4% diet DM) of FO. Dry matter intake was not affected with feeding FO to dairy cows at 4% of the diet DM (Bu et al., 2007). In contrast, Martin et al. (2008) observed a 25% decrease in DMI when dairy cows were supplemented with FO at 5.7% of the diet DM. According to Chilliard et al. (1993), supplemental fat may negatively impact DMI through the following mechanisms: (1) an increase in rumination time due to negative effects on ruminal digestion; and (2) a slowdown of rumen emptying due to a metabolic effect of long chain fatty acids considering that it is possible that fatty acids intake may have a negative feedback on voluntary intake via inhibition of rumino-reticular motility (Chilliard et al., 1993). In both situations, ruminal fill could result in a satiety effect depressing DMI (Chilliard et al., 1993). Despite the reduction in DMI observed in cows fed FM diets, milk yield did not differ between cows fed the -CTRL diet and those fed the SUCR and OIL diets. However, a negative associative effect was observed for milk production ($P = 0.05$) when FM was supplemented with sucrose and FO (i.e., COMBO diet). Depressed

milk yield with feeding COMBO can be explained by the decreased DMI and apparent total-tract digestibilities of DM, OM, and ADF as discussed earlier. Additionally, in general, the FM-based diets fed in this study likely had less starch compared with the control diet due to less ground corn and could have resulted in less fermentable energy from starch leading to decreased milk yield in cows fed the COMBO diet. Energy corrected milk ($P = 0.001$) and 4% FCM ($P = 0.001$) were both lower in cows fed the OIL and COMBO compared to those fed -CRTL and SUCR. These results can be explained by the reduced milk fat yield in cows fed -CRTL and SUCR diets. Feed efficiency expressed as milk yield/DMI did not differ among experimental diets and was 1.47, 1.51, 1.55 and 1.53 in cows fed -CRT, SUCR, OIL and COMBO diets, respectively.

The concentration and yield of milk fat decreased ($P < 0.001$) when FO was added to FM (Table 11). The proposed mechanism by which supplemental fat can result in milk fat depression suggests that ruminal function is altered by dietary supplementation with unsaturated fatty acids and creates intermediate fatty acids products that inhibit milk fat synthesis (Bauman and Griinari, 2003). An intermediate that can be produced with unsaturated fatty acids supplementation is *trans*-10, *cis*-12 18:2, which has been shown to cause a 25% reduction in milk fat yield when dosed into the rumen at 3.5 g/d (Baumgard et al., 2001). Previous research on the effects of supplementing FO on milk fat content have not been consistent. For instance, in the study done by Benchaar et al. (2012) feeding incremental amounts of FO had no effects on concentration and yield of milk fat. By contrast, milk fat content decreased when FO was supplemented to dairy cows at 4% of the DM (Pi et al.,2016) and at 2.0 % of DM (Flachowsky et al., 2016). No effects of treatments were observed regarding concentrations and yield of milk true protein, and concentration of milk lactose (Table 11). However, lactose yield ($P = 0.08$) and MUN ($P = 0.09$) tended to decrease in the COMBO diet (Table 11). Because the dietary concentration of CP was

relatively similar across experimental diets (Table 10), the reduction in N intake appears to partially explain the observed tendency for decrease in MUN.

Apparent total-tract digestibility of nutrients is presented in Table 12. Digestibility of DM ($P = 0.01$) and OM ($P < 0.01$) were lower in cows fed FM diets (i.e., SUCR, OIL, COMBO) than in those offered the –CRTL treatment. Decreased apparent total tract digestibility of DM, OM suggests that increased fat, more specifically increased PUFA and replacement of starch by sugar in FM-based diets may have affected ruminal function negatively. Flaxseed meal-based supplemented with FO (i.e. OIL and COMBRO) had greater concentration of rumen available fat compared to the remaining diets (i.e. SUCR and -CRTL) (Table 10), which can partially explain the observed decrease in DM and OM digestibilities. The apparent total-tract digestibility of ADF was greatest in –CRTL, intermediate in SUCR, and lowest in OIL and COMBO (P = 0.05; Table 12). Depressed ADF digestibility with the addition of FO suggests that increased intake of PUFA may have negatively impacted the ruminal function. The effect of PUFA on function of ruminal microbes was tested in vitro by Maia et al. (2007). Among 24 bacterial species studied, 11 had decreased growth when α linolenic acid was added. Alpha Linolenic acid was shown to have greater toxicity to cell growth than linoleic acid (Maia et al., 2007). Flaxseed oil contains high levels of α-linolenic acid (NRC, 2001; Schroeder et al., 2004) and decreased DM, OM, and ADF digestibilities could be attributed to the negative impact of α-linolenic acid on ruminal fermentation. No differences across treatments were observed for the apparent total-tract digestibilities of NDF and CP, which averaged 51.21 and 69.35%, respectively (Table 12).

Milk EL and Plasma TBARS

As expected, the concentration and yield of milk EL were both greater ($P < 0.001$) in cows fed FM diets (i.e., SUCR, OIL, COMBO) than those fed soybean meal (i.e., –CRTL treatment) (Table 11 and Figure 6). Increased concentration and yield of milk EL in cows fed SUCR, OIL, and COMBO is explained by the presence of SDG in FM (Table 9). In fact, flaxseed is the richest source of SDG, which is a precursor for the synthesis of mammalian lignans by the gut microbiota of humans (Thompson et al., 1991; Gaya et al., 2016) and ruminants (Gagnon et al., 2009; Zhou et al., 2009). Our results agree with previous studies that showed improvement on milk EL concentration when FM was fed up to 15% of the diet DM to dairy cows (Petit and Gagnon, 2009; Brito et al., 2015). Schogor et al. (2014) reported that *Prevotella spp*. were the main converters of SDG to secoisolariciresinol, a lignan-derived metabolite that is further metabolized to ED and EL. Therefore, dietary changes that favor *Prevotella spp* growth in the rumen could potentially improve the conversion of SDG to EL in the rumen, ultimately increasing the output of lignans in milk. *Prevotella spp* are also capable of utilizing starch, other noncellulosic polysaccharides, and simple sugars as energy sources yielding succinate as the major end product of ruminal fermentation (Purushe et al., 2010). Limited information is available regarding the relationship between *Prevotella spp* and dietary fat and sugar supplementation. Huws et al. (2010) reported no effect of fish oil supplementation on *Prevotella spp* relative abundance in beef steers. However, *Prevotella spp* relative abundance was affected by an interaction between starch level and sunflower oil in non-lactating Holstein cows (Zened et al.; 2013). Switching from a low starch to a high starch diet slightly decreased the relative abundance of *Prevotella spp,* whereas switching from a low-starch plus oil to a high-starch plus oil diet resulted in a 2-fold increase in *Prevotella spp*. relative abundance according

to Zened et al. (2013). This high relative abundance of *Prevotella spp*. with feeding the high-starch plus oil diet could be related to their ability to degrade a wide variety of substrates and their resistance to unsaturated fatty acids. More recently, Li et al. (2015) showed that when beef steers were supplemented (DM basis) with 4% FO the genus *Prevotella* dominated the ruminal bacterial community. However, milk EL concentration and yield did not change in cows fed FM in the current study, suggesting that neither sucrose nor FO alone or their combination (i.e., the COMBO diet) was able to elicit substantial shifts in the ruminal microbiota favoring an increase in SDG metabolism towards EL production. It has been reported that compared with ground corn, feeding liquid molasses increased the concentration and yield of milk EL in dairy cows offered grass hay plus 15% FM (Brito et al., 2015), thus suggesting that sucrose, the major sugar found in molasses, may select for ruminal microorganisms with increased capacity to convert FM-SDG to EL. However, in the present study milk EL did not differ in FM-based diets supplemented with sucrose (i.e. SUCR and COMBO) and FO (i.e. OIL).

Lipid peroxidation in plasma was determined by production of TBARS which is detailed in Table 12. There was no effect of treatments on plasma concentration of TBARS (Table 12). Studies have investigated the effects of FM on protection against oxidation in dairy cows. De Marchi et al. (2017) found no effects of FM supplementation on plasma and milk concentration of TBARS in dairy cows fed a source of n-6 fatty acids that bypasses the rumen and concluded that FM supplementation did not protected cows from lipoperoxidation. In a similar study, Lima et al. (2014) reported no changes for plasma and milk TBARS concentration when FM was fed to cows receiving FO infusion in the abomasum. Comparison of our results to studies cited previously is limited since in this study cows were fed FO, which is available for ruminal degradation whereas, oil sources used in the studies done by De Marchi et (2017) and Lima et al.

(2014) were unavailable in the rumen. Additionally, cows used in this study were not likely to be in oxidative stress which limits drawing a conclusion regarding a possible antioxidant protective effect of FM-based diets.

Nitrogen Metabolism

Effects of treatments on urinary excretion of nitrogenous metabolites are presented in Table 12. Urinary volume was lowest in cows fed the COMBO diet ($P = 0.01$). Reduced urinary volume observed for cows fed the COMBO diet is consistent with the lowest intake of K ($P < 0.001$) and Na ($P = 0.01$) reported in this treatment (Table 11). According to Bannink et al. (1999), intake and urinary excretion of K, Na, and N explained, respectively, 85.8 and 89.8% of the variance when regressed against actual urinary volume in lactating dairy cows. Eriksson and Rustas (2014) observed a positive linear relationship between K intake and urinary volume and a negative linear relationship between K intake and MUN in dairy cows. Spek et al. (2012) showed that feeding incremental amounts of Na to dairy cows increased linearly the urinary volume and total urinary N excretion. According to Spek et al. (2012) and Eriksson and Rustas (2014), elevated Na and K intake leads to increased renal glomerular filtration rate and a temporary increase in the urinary excretion of urea N, which ultimately drops MUN and PUN. In the present study, MUN tended to be lower in cows fed COMBO compared with the other 3 diets (Table 11), but PUN was not different across treatments (Table 12). No treatment effects were observed for the urinary excretion of allantoin (mean = 350 mmol/d), uric acid (mean = 72.2 mmol/d), PD (422 = mmol/d), total N (mean = 209 g/d), and urea N (mean = 135. g/d). However, total urinary N excretion, expressed as a proportion of N intake, was greatest in OIL, intermediate in COMBO, and lowest in –CRTL and SUCR diets. It is possible that diets

containing oil (i.e. OIL and COMBO diets) had less fermentable energy as starch (less GRC) compared with the –CRTL and SUCR diets leading to a lower N efficiency.

CONCLUSIONS

Feeding dairy cows diets containing (DM basis) 15% of FM increased the concentration and yield of milk EL compared with feeding soybean meal (-CRTL diet). No difference in milk EL was observed when FM was supplemented with either sucrose or FO alone or their combination (COMBO diet), suggesting no synergistic effects of sucrose and FO in the conversion of SDG to EL in the rumen. Further research is needed to better understand how FM-SDG metabolism is affected by changes in diversity and function of the ruminal microbiome.

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Table 9. Nutritional composition of feedstuffs (mean ± SD) used in the experimental diets (% of DM, unless otherwise noted)

	Forages		Concentrates			
Item	Corn silage	Haylage	Flaxseed meal	Ground corn	Soybean meal	Rumen bypass protein[2]
DM, % of fresh matter	40.7 ± 2.42	48.3 ± 2.00	96.3 ± 0.26	92.7± 3.63	93.7	92.8
CP	7.70± 0.24	18.0 ± 0.41	37.1 ± 0.17	9.35 ± 0.36	47.0	43.3
Soluble protein, % of CP	65.2 ± 2.75	53.5 ± 1.73	49.2 ± 8.34	21.2 ± 0.50	31.0	13.0
NDF	36.3 ± 2.24	53.3 ± 2.56	36.3 ± 2.76	8.70 ± 0.80	14.1	31.0
ADF	19.6 ± 1.44	34.5 ± 1.88	17.3 ± 0.97	3.80 ± 0.58	10.3	17.7
Acid detergent lignin	2.72 ± 0.51	7.07 ± 1.06	6.90 ± 0.88	1.40 ± 0.09	4.30	7.80
Ether extract	4.00 ± 0.39	5.25 ± 0.37	11.6 ± 0.13	4.00 ± 0.08	2.07	3.40
Ash	4.06 ± 0.20	8.48 ± 0.38	6.45 ± 0.10	1.40 ± 0.02	7.50	7.75
NFC	48.0 ± 2.78	15.0 ± 2.84	8.41 ± 2.80	76.5 ± 1.04	28.6	14.5
SDG[1]	-	-	1.30 ± 0.26	-	-	-
Ca	0.16 ± 0.01	0.80 ± 0.09	0.53 ± 0.05	0.01 ± 0.005	0.67	0.72
P	0.28 ± 0.02	0.34 ± 0.01	0.95 ± 0.06	0.33 ± 0.01	0.79	1.06
Mg	0.13 ± 0.01	0.29 ± 0.03	0.60 ± 0.05	0.12 ± 0.005	0.34	0.54
K	1.01 ± 0.04	2.22 ± 0.25	1.23 ± 0.10	0.46 ± 0.03	2.55	1.77
S	0.09 ± 0.005	0.25 ± 0.03	0.50 ± 0.03	0.11 ± 0.008	0.01	0.72
Na	<.001	0.05 ± 0.02	0.10 ± 0.01	0.004 ± 0.002	0.45	0.10
Fe, mg/kg	81.0 ± 10.8	243 ± 167	224 ± 12.8	27.5 ± 2.38	131	210
Cu, mg/kg	4.00 ± 0	10.2 ± 1.71	20.7 ± 1.89	1.70 ± 0.50	19.0	9.00
Mn, mg/kg	8.75 ± 0.95	57.2 ± 15.2	52.5 ± 5.06	5.50 ± 0.57	52.0	57.0
Zn, mg/kg	19.2 ± 1.70	30.7 ± 5.31	81.7 ± 5.85	21.2 ± 0.95	56.0	56.0
Mo, mg/kg	0.77± 0.45	2.77 ± 0.74	0.95 ± 0.17	0.35 ± 0.13	4.60	2.60

[1] Secoisolariciresinol diglcoside (SDG), analyzed according to Muir and Westcott (2000).

[2] AminoMax Pro Bypass Protein: plant protein sources that are consistently high in ruminal bypass and amino acid bioavailability mainly, soybean meal and canola meal.

Table 10. Ingredient and nutritional composition (% of DM, unless otherwise noted) of the experimental diets

	Experimental diets[1]			
Item	-CTRL	SUCR	OIL	COMBO
Ingredient composition				
Corn silage	40.0	40.0	40.0	40.0
Haylage	20.0	20.0	20.0	20.0
Sugar	0.00	5.00	0.00	5.00
Ground corn	23.1	10.7	15.4	10.2
Flaxseed meal	0.00	15.0	15.0	15.0
Soybean meal	8.00	0.00	0.00	0.00
Flaxseed oil	0.00	0.00	3.00	3.00
Rumen bypass fat[2]	2.00	2.50	0.00	0.00
Rumen bypass protein[3]	4.00	4.00	4.00	4.00
Minerals-vitamins premix[4]	2.00	2.00	2.00	2.00
Nutritional composition[5]				
DM, % of fresh matter	51.6	51.3	51.0	51.0
CP	17.0	17.2	17.3	17.2
NDF	29.5	32.8	33.2	32.8
ADF	17.2	18.5	18.9	18.4
Acid detergent lignin	3.49	4.01	4.08	4.00
NFC[6]	42.7	35.1	37.2	36.8
Ether extract	3.92	4.96	5.15	4.94
Ash	4.56	4.75	4.82	4.74
Ca	0.71	0.73	0.73	0.73
P	0.39	0.44	0.42	0.42
K	1.23	1.18	1.16	1.15
Na	0.20	0.21	0.21	0.21
NE_L,[7]Mcal/kg of DM	1.60	1.60	1.56	1.60

[1]Experimental diets: -CTRL; 8% SBM plus 23% ground corn); 15% FM +10.7% ground corn + 5% sucrose (SUCR); 15% FM + 15.4% ground corn + 3% FO (OIL); and 15% FM +10.2% ground corn + 5% sucrose + 3% FO (COMBO).

[2]BergaFat F-100: rumen bypass fats for ruminants. Fractionated palm fatty acids with high percentage of palmitic acid.

[3]AminoMax Pro Bypass Protein: plant protein sources that are consistently high in ruminal bypass and amino acid bioavailability mainly, soybean meal and canola meal.

[4]Mineral and vitamin mix provided on as fed basis: 11.3% Ca, 1.76% P, 5.98% Mg, 6% K, 3% S, 15 mg/kg Co, 650 mg/kg Cu, 50 mg/kg I, 1,200 mg/kg Mn, 8.97 mg/kg Se, 3,700 mg/kg Zn, and 87.1 KIU/kg vitamin A.

[5]The nutritional composition of the diets was calculated based on the individual nutritional composition of the dietary ingredients as reported in Table 9.

[6]NFC = 100 – [CP + (NDF – NDICP) + fat + ash]

[7]Determined using the NRC (2001) including actual feed nutritional composition and animal variables (i.e., DMI, milk yield and composition, DIM, and BW).

Table 11. Milk yield and composition and DMI of Holstein cows fed flaxseed meal-based diets supplemented with sucrose and flaxseed oil alone or their combination

	Experimental diets[1]					
Item	-CTRL	SUCR	OIL	COMBO	SEM	*P*-value
DMI, kg/d	24.6[a]	23.7[b]	22.5[c]	22.0[c]	1.07	<0.001
N intake, g/d	675[a]	660[a]	625[b]	614[b]	11.5	<0.001
K intake, g/d	302[a]	273[b]	265[b]	253[c]	12.5	<0.001
Na intake, g/d	49.1[a]	49.4[a]	47.2[b]	46.2[b]	2.20	0.01
SDG intake, g/d[2]	-	50.0	48.0	47.8	4.72	<0.001
Milk yield, kg/d	36.2[a]	35.7[a]	34.8[ab]	33.4[b]	2.17	0.05
4% FCM[3], kg/d	34.4[a]	34.1[a]	29.4[b]	27.4[b]	2.03	<0.001
ECM[4], kg/d	36.3[a]	36.4[a]	32.0[b]	30.0[b]	2.17	<0.001
Milk yield/DMI, kg/kg	1.47	1.51	1.55	1.53	0.11	0.14
4% FCM/DMI, kg/kg	1.39	1.43	1.30	1.24	0.13	0.09
ECM/DMI, kg/kg	1.47	1.53	1.42	1.36	0.10	0.25
Milk EL, n*M*	76.8[a]	293[b]	332[b]	338[b]	49.3	<0.001
Milk EL, mg/d	0.81[a]	3.31[b]	3.51[b]	3.50[b]	0.60	<0.01
Milk fat, %	3.66[a]	3.69[a]	2.96[b]	2.83[b]	0.12	<0.001
Milk fat, kg/d	1.33[a]	1.32[a]	1.03[b]	0.93[b]	0.08	<0.001
Milk true protein, %	2.98	2.94	2.92	2.96	0.07	0.66
Milk true protein, kg/d	1.01	1.04	1.01	0.97	0.07	0.63
Milk lactose, %	4.89	4.85	4.91	4.90	0.09	0.23
Milk lactose, kg/d	1.79	1.75	1.72	1.65	0.11	0.08
MUN, mg/dL	12.0	13.2	11.3	10.7	1.11	0.09

[1]Experimental diets: -CTRL; 8% SBM plus 23% ground corn); 15% FM +10.7% ground corn + 5% sucrose (SUCR); 15% FM + 15.4% ground corn + 3% FO (OIL); and 15% FM +10.2% ground corn + 5% sucrose + 3% FO (COMBO).

[2]SDG intake from flaxseed meal only.

[3]4% FCM = [0.40 × milk yield (kg/d)] + [15 × milk fat yield (kg/d)] (Gaines and Davidson, 1923).
[4]ECM = [0.327 × milk yield (kg/d)] + [12.95 × fat yield (kg/d)] + [7.2 × protein yield (kg/d)] (Orth, 1992).
[a,b,c]Different letters in the same row indicate significant differences at $P \leq 0.05$; trends were declared at $0.05 < P \leq 0.10$.

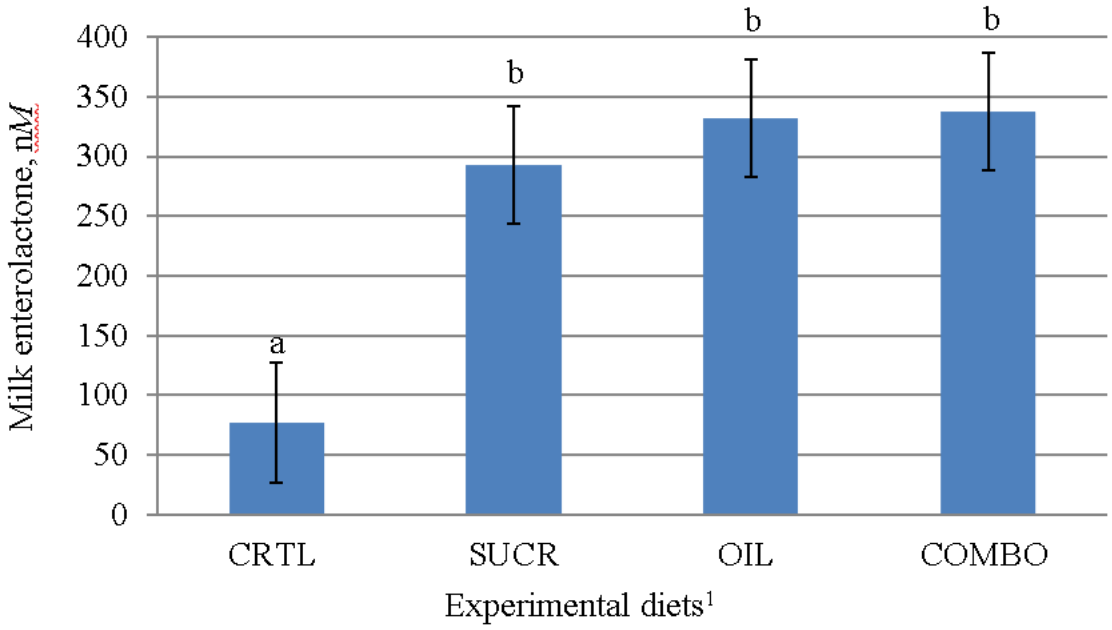

Figure 6. Effects of feeding flaxseed meal-based diets supplemented with sugar or flaxseed oil alone or their combination on milk concentration of enterolactone (n*M*) in Holstein dairy cows. Data are presented as least square means ± SEM. Different letters indicate significant differences at $P \leq 0.05$ and trends at $P \leq 0.10$. [1]Experimental diets: -CTRL; 8% SBM plus 23% ground corn); 15% FM +10.7% ground corn + 5% sucrose (SUCR); 15% FM + 15.4% ground corn + 3% FO (OIL); and 15% FM +10.2% ground corn + 5% sucrose + 3% FO (COMBO).

Table 12. Effects of feeding flaxseed meal-based diets supplemented with sucrose and flaxseed oil alone or their combination on apparent total-tract digestibility of nutrients, blood parameters (TBRAS and PUN) and urinary excretion of nitrogenous metabolites in Holstein cows

	Experimental diets[1]					
Item	-CRTL	SUCR	OIL	COMBO	SEM	*P*-value
Apparent total-tract digestibility						
DM, % of DMI	72.4[a]	68.8[b]	68.0[b]	69.3[b]	1.48	0.01
OM, % of OM intake	72.6[a]	66.9[b]	67.8[b]	67.1[b]	1.59	<0.01
NDF, % of NDF intake	52.3	52.2	49.1	51.2	2.85	0.31
ADF, % of ADF intake	55.5[a]	52.6[ab]	48.7[b]	50.8[b]	2.97	0.05
CP, % of CP intake	71.1	68.0	69.1	69.2	1.40	0.15
Blood parameters						
PUN, mg/dL	20.41	20.58	22.71	21.87	1.22	0.33
TBARS[2]	11.83	14.08	13.38	15.77	2.58	0.31
Urinary volume and excretion						
Urine, L/d	49.2[a]	51.2[a]	47.8[a]	40.8[b]	2.52	0.01
Creatinine, m*M*	3.70[a]	3.47[a]	3.77[a]	4.41[b]	0.17	0.01
Uric acid, mmol/d	74.6	78.3	65.3	70.8	7.94	0.55
Allantoin, mmol/d	348	361	351	341	32.0	0.92
Total PD[3], mmol/d	423	439	417	412	11.6	0.89
Total N, g/d	198	217	227	195	22.1	0.15
Total N, % of N intake	29.7[a]	31.8[a]	36.5[b]	32.3[ab]	2.53	0.05
Urea N, g/d	127	136	141	134	22.7	0.62
Urea N, % total urinary N	57.1	54.2	54.9	60.5	4.40	0.59

[1] Experimental diets: -CTRL; 8% SBM plus 23% ground corn); 15% FM +10.7% ground corn + 5% sucrose (SUCR); 15% FM + 15.4% ground corn + 3% FO (OIL); and 15% FM +10.2% ground corn + 5% sucrose + 3% FO (COMBO).
[2] Concentration of thiobarbituric acid reactive substances (TBARS) expressed in terms of malondialdehyde (MDA) equivalents (μ*M*).
[3]Total purine derivatives (PD) = uric acid + allantoin.

CHAPTER IV: THE MAMMALIAN LIGNAN ENTEROLACTONE IS ABSORBED BY NEWBORN DAIRY CALVES FED ENTEROLACTONE ENRICHED MILK

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SHORT COMMUNICATION

Lignans are polyphenolic, phytoestrogenic compounds known to elicit a wide range of biological effects, including weak estrogenic, antiestrogenic, antioxidant, anti-inflammatory, anticarcinogenic, and cardioprotective activities (Adolphe et al., 2010; Imran et al., 2015). Flaxseed (*Linum usitatissimum* L.) is the richest source of the lignan secoisolariciresinol diglycoside (**SDG**), which is a precursor for the synthesis of the mammalian lignans enterolactone (**EL**) and enterodiol (**ED**) by the gut microbiota of humans (Gaya et al., 2016) and ruminants (Gagnon et al., 2009). Feeding incremental amounts of flaxseed meal to dairy cows linearly increased the milk concentration of EL, but no ED was detected in milk (Petit et al., 2008). Therefore, EL-enriched milk has the potential to be used as a source of EL for humans not only because milk is consumed by a large part of the world population, but also due to a poor and variable intake of plant lignans worldwide, including in the United States (de Kleijn et al., 2001).

Newborn calves often experience diarrhea, respiratory diseases, and oxidative stress, which contribute to high rates of morbidity and mortality during the first weeks of life (Inamani et al., 1999; Gaál et al., 2006; Uetake, 2013). In addition, poor colostrum quality is associated with low concentration of antioxidants (Maciej et al., 2015), suggesting that feeding EL-enriched milk to newborn and pre-weaned calves may be a viable strategy to mitigate oxidative stress. In

newborn calves, suckling stimulates the reflex closure of the esophageal groove so that milk or milk replacer (**MR**) bypass the reticulo-rumen down to the abomasum. Thus, calves may be used as a model to make inferences about the pharmacokinetics of EL in simple-stomach mammals including humans. We hypothesized that compared with calves fed MR, those fed EL-enriched milk would have increased area under the curve (**AUC**), as well as greater maximum plasma concentration ($\mathbf{C_{max}}$) of EL and faster time to reach C_{max} ($\mathbf{T_{max}}$). The objective of this study was to determine the pharmacokinetics of EL from MR or EL-enriched milk consumed by newborn Holstein calves.

This experiment was conducted at the University of New Hampshire Fairchild Dairy Teaching and Research Center (Durham, NH) from May to August 2015 and was approved by the University of New Hampshire Institutional Animal Care and Use Committee (protocol no. 15303). Twenty Holsteins calves (n = 10 males and 10 females) were used from birth to d 7 of life. Calves were removed from dams immediately after birth and prior to nursing, weighed, navel-dipped with 7% iodine (vol:vol), and placed in individual pens (1 × 2.15 m) located in an enclosed calf room. The 10 calves (n = 6 males and 4 females) born from multiparous cows received 4 L of colostrum using nipple bottles, with the first 2 L fed immediately after they were moved to the pens and the remaining 2 L within the first 24 h of life. The 10 calves (n = 4 males and 6 females) born from primiparous cows were fed 4 L of stored colostrum from multiparous cows when available or colostrum replacer (Ultra start 150 plus; Milk Products LLC, Chilton, WI) split in 2 daily allotments of 2 L each as done for calves born from multiparous cows. When colostrum or colostrum replacer was completely refused or not fully consumed voluntarily within approximately 15 min after offering the meal, refusal was administered via an esophageal tube to

ensure uniform consumption among animals. Calves had free access to water and no access to starter grain while enrolled in the study.

From d 2 to d 4 of life, nipple bottles were used to feed 4 L/d of non-medicated MR (Calf care all-milk 22-20; Poulin Grain, Newport, VT) to all calves in 2 daily allotments (0700 and 1900 h) by mixing 300 g of MR powder plus 2 L of warm tap water following standard operation procedures of our dairy facility. On d 5 of life, calves were randomly assigned to 1 of 2 treatments: Low milk EL (**L-EL**; n = 5 females and 5 males) or High milk EL (**H-EL**; n = 5 females and 5 males). Calves assigned to the H-EL treatment had MR substituted for 2 L of EL-enriched milk during the morning feeding on d 5 of life, while L-EL calves continued to receive 2 L of MR per feeding until d 7. Administration of MR resumed at 1900 h on d 5 for H-EL calves and continued through d 7. All calves completely consumed the MR or EL-enriched milk within 5 min after the meal was offered. The EL-enriched milk used in the current study was collected over 3 consecutive afternoon milkings (total = 28 kg) from 1 multiparous Jersey cow fed a TMR containing (DM basis) 15% flaxseed meal and 12% liquid molasses (Ghedini et al., 2016). Milk was stored at -20°C in 3-L plastic bottles for at least 90 d before being administered to the calves using nipple bottles. The concentration of EL (mean ± SD) averaged 123 ± 6.53 nmol/L and 481 ± 65 nmol/L for MR and EL-enriched milk, respectively. The health status of all calves was evaluated daily while they were enrolled in the study (i.e., 7 d) by monitoring signs of illness including lethargy, weakness, decreased appetite, fever, abnormal fecal consistency, cough, ocular or nasal discharge, and drooping ears using a calf health scoring chart (https://www.vetmed.wisc.edu/dms/fapm/fapmtools/8calf/calf_health_scoring_chart.pdf).

Blood samples were taken from the jugular vein on d 5 of life before (0 h) and at 0.5, 1, 1.5, 2, 2.5, 3, 4, 6, 8, 10, 12, 24, and 48 h after oral administration of MR or EL-enriched milk

using 10-mL vacutainer tubes containing K_3-EDTA (Covidien, Minneapolis, MN). After collection, tubes were immediately transported to the laboratory and centrifuged (3,300 × g) for 20 min at 4°C. Aliquots (1.8 µL) of plasma were stored at -80ºC until EL analysis. Samples (40 mL) of MR and EL-enriched milk were taken during the morning feeding on d 5 and stored at -20°C for later EL analysis. Enterolactone in plasma, MR, and EL-enriched milk was extracted and hydrolyzed [β-glucuronidase/arylsulfatase from *Helix pomatia* (Roche-Diagnostics; Laval, QC, Canada)] according to procedures described previously (Gagnon et al., 2009). Enterolactone was analyzed colorimetrically (UV/visible spectrophotometer set at a wavelength of 405 nm) in quadruplicates using a competitive enzymatic immunoassay (assay kit no. 500520; Cayman Chemical Co., Ann Arbor, MI) that recognizes both enantiomeric forms of EL. Plasma EL values were corrected by baseline concentrations of EL (i.e., 0-h blood sampling).

The AUC between 0 and 12 h ($AUC_{0\text{-}12\ h}$) after oral administration of MR or EL-enriched milk on d 5 of life was determined according to the trapezoidal rule (Phillips and Taylor, 1973). Both $C_{max\ (0\text{-}12\ h)}$ and $T_{max\ (0\text{-}12\ h)}$ were determined from individual baseline corrected plasma concentration time curves (Maciej et al., 2015). The apparent efficiency of absorption (**AEA**) of EL between 0 and 12 h was calculated assuming no change in plasma volume from d 1 to d 5 of life using the following equation (Quigley et al., 1998):

$$AEA_{0\text{-}12\ h},\ \% = [\text{plasma EL (mg/L)} \times \text{BW (kg)} \times 0.092 \div \text{EL intake (mg)}] \times 100$$

The experiment was analyzed as a randomized complete block design with repeated measures over time [i.e., $AUC_{0\text{-}12\ h}$, $C_{max\ (0\text{-}12\ h)}$, and $T_{max\ (0\text{-}12\ h)}$] using the MIXED procedure of SAS (Version 9.4; SAS Institute Inc., Cary, NC). Calves were blocked by date of birth yielding a total of 10 blocks with 2 animals/block. The random effect of block, as well as the fixed effects of treatment, time of blood sampling, and covariate measurements (i.e., initial BW and 0-h

plasma concentration of EL) were included in the statistical model. The random effect of calf within treatment was used as the error term. The covariate structure heterogeneous first order autoregressive yielded the lowest Bayesian Information and was retained in the final model. Body weight at birth, EL intake, plasma concentrations of EL (0 h, 12 h, 24 h, and 48 h), and $AEA_{0\text{-}12\ h}$ were analyzed without the repeated statement in the model. Data are reported as LSM and SEM. Significance was declared at $P \leq 0.05$ and trends at $0.05 < P \leq 0.10$.

All calves were born spontaneously without signs of illnesses. The concentration of EL averaged 123 nmol/L in MR and was about 80 and 26% greater than the EL content found in whole milk of Holstein (Petit and Gagnon, 2009; Petit et al., 2009) and Jersey (Brito et al., 2015) cows not supplemented with flaxseed meal, thus indicating that EL is more concentrated in MR than whole milk. Other non-SDG plant lignans such as matairesinol, pinoresinol, and lariciresinol have been shown to be converted to EL (Heinonen et al., 2001). Furthermore, the mean duodenal flow and fecal excretion of secoisolariciresinol, matairesinol, ED, and EL were greater than the intake of these metabolites, implying that plant lignans besides matairesinol and secoisolariciresinol are precursors of EL production in the rumen (Njåstad et al., 2014).

Initial BW and EL pharmokineticts results are presented in Table 13. Body weight at birth did not differ ($P = 0.81$) between treatments. As expected, EL intake was 314% greater ($P < 0.001$) in H-EL than L-EL calves. The $AUC_{0\text{-}12\ h}$ for plasma EL was greater ($P < 0.001$) in calves fed H-EL compared with those fed L-EL and averaged 26.0 and 4.30 nmol/L × h, respectively. Similarly, $C_{max\ (0\text{-}12\ h)}$ was greater ($P < 0.001$) in H-EL (5.06 nmol/L) vs. L-EL calves (1.95 nmol/L). In addition, $T_{max\ (0\text{-}12\ h)}$ was reached 13 min faster ($P < 0.001$) in calves administered H-EL (4.31 h) than L-EL (4.44 h). Plasma concentrations of EL were greater at 12 h (3.21 vs. 1.16 nmol/L; $P < 0.001$), 24 h (2.67 vs. 1.07 nmol/L; $P < 0.001$), and 48 h (2.05 vs.

1.07 nmol/L; $P < 0.001$) when feeding H-EL vs. L-EL, respectively. However, no significant difference between treatments was observed for the baseline concentration (i.e., 0-h blood sampling) of EL in plasma. These results collectively suggest that EL was absorbed in the intestines of newborn dairy calves consuming MR or EL-enriched milk. As newborn calves experience high rates of oxidative stress, morbidity, and mortality (Inamani et al., 1999; Gaál et al., 2006; Uetake, 2013), administration of EL-enriched milk may be used as a feeding strategy with the potential to improve calf health during the first weeks of life.

The plasma concentration of EL in calves assigned to L-EL returned to baseline 12 h after oral administration of MR, but not in calves administered H-EL (Figure 7). Maciej et al. (2015) reported that the plasma concentration of total flavonols, which are polyphenols with potential health-promoting properties, did not return to baseline 12 h after oral administration of quercetin aglycone or rutin to 2-d old German Holstein calves. However, the plasma concentration of total flavonols returned to baseline 48 h past oral dose (Maciej et al., 2015), but that of EL from EL-enriched milk did not (Figure 7). Whereas quercetin aglycone or rutin was fed as a single dose (Maciej et al., 2015), the oral administration of MR to H-EL calves resumed at 1900 h on d 5 of life. This likely influenced the plasma concentration of EL at 48 h (d 7 of life) as the concentration of EL in MR averaged 123 nmol/L.

It has been shown in vitro using human colon epithelial cells (Jansen et al., 2005) or rat and human intestinal and liver microsomes (Lin et al., 2013) that EL and ED are conjugated as glucuronides and sulfates. Conjugated EL and ED undergo extensive first-pass metabolism and enterohepatic recirculation (Jansen et al., 2005; Lin et al., 2013), as well as deconjugation by bacterial β-glucuronidases and sulfatases followed by reabsorption (Setchell and Adlercreutz 1988). In lactating dairy cows, absorption of EL and ED appears to occur in the rumen (Gagnon

et al., 2009) and intestines (Njåstad et al., 2014), likely as conjugated forms. Therefore, it is conceivable that most EL found in ruminants' milk is conjugated with glucuronides and sulfates.

In the present study, newborn calves were able to absorb EL, indicating that they may have active β-glucuronidases produced by the intestinal microbiota or that absorption of EL was independent of enzymatic action. The activity of β-glucuronidase in humans has been attributed to intestinal-dominant bacteria belonging to *Bacteroides*, *Bifidobacterium*, *Eubacterium*, and *Ruminococcus* genera (Hawkesworth et al., 1971; Akao et al., 2000). In ruminants, the presence of *Bacteroides* in the cecum and feces of Jersey calves has been reported as early as d 2 of life (Smith, 1965). Increased small intestinal tissue-attached *Bifidobacterium* 6 h after birth has been reported in Holstein calves fed heat-treated colostrum (Malmuthuge et al., 2015). *Bifidobacterium* increased in fecal samples of calves between d 3 and d 7 of life, and active fecal β-glucuronidase was detected in all *Bifidobacterium*-positive calves (Rada et al., 2006). However, in contrast to non-ruminants, studies conducted with lactating dairy cows suggest marginal involvement of β-glucuronidase in the absorption of EL (Gagnon et al., 2009; De Marchi et al., 2016)

The $AEA_{0\text{-}12\ h}$ of EL was < 2% and tended ($P = 0.08$) to be lower in H-EL (1.31%) than L-EL calves (1.85%) as shown in Table 13. Similarly, the oral bioavailability of ED averaged < 1% in Wistar rats (Mukker et al., 2015). Low bioavailability of enterolignans may be attributed to poor intestinal permeability or high first-pass metabolism and systemic clearance (Mukker et al., 2015). Guglielmini et al. (2012), reported an association between serum EL concentration ≥ 10 nmol/L and decreased mortality risk (all-cause and breast cancer-specific) in women after breast cancer surgery. Milk concentration of EL averaged 395 nmol/L across 2 studies where Jersey cows received 15% of the diet DM as flaxseed meal (Brito et al., 2015; Ghedini et al.,

2016). Thus, 1 daily serving (250 mL) of EL-enriched milk with a concentration of 395 nmol/L EL would result in 1.3 nmol/L of EL in plasma assuming an $AEA_{0\text{-}12\ h}$ of 1.31% (Table 13). This implies that EL-enriched milk needs to be consumed in combination with other lignan-rich foods to reach EL concentration in blood that has been linked to decreased mortality and cancer risks (Guglielmini et al., 2012). However, our inferences should be interpreted cautiously as calves in the present study were fed a liquid diet, which may have increased the passage of milk through the gastrointestinal tract ultimately limiting EL absorption. Furthermore, the developing intestinal microbiota of newborn calves is likely unfit to fully metabolize conjugated EL. Thus, it is conceivable that the gut microbiota of adult humans would be able to metabolize EL-enriched milk more efficiently than that of newborn dairy calves.

Our hypothesis that the plasma AUC of EL would be greater in calves fed EL-enriched milk (H-EL treatment) compared with those fed MR (L-EL treatment) was confirmed, indicating that EL-enriched milk can be potentially used as a source of EL to pre-weaned calves. Further research is needed to better understand the absorption and transportation mechanisms of EL along the intestinal tract of newborn calves in the context of a developing gut microbiota. Dose-response studies, as well as factorial experiments feeding whole milk or MR supplemented or not with SDG should be also conducted in the future. Based on the pharmacokinetics data of EL derived from newborn dairy calves, humans should consume EL-enriched milk in combination with other lignan-rich foods to capitalize on potential health benefits. However, human-specific studies should be conducted due to the limitations of using newborn calves as a translational model for humans.

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Table 13. Least square means for BW at birth, and intake and pharmacokinetics of enterolactone (EL) in newborn Holstein caves fed milk replacer (Low-EL; n = 10) or EL-enriched milk (High-EL; n = 10)

	Treatments			
Item[1]	Low-EL	High-EL	SEM	*P*-value[2]
BW, kg	41.3	42.2	2.39	0.81
EL intake, μg	70.0	290	8.00	<0.001
Plasma $AUC_{0\text{-}12\ h}$[3], nmol/L × h	4.30	26.0	1.69	<0.001
Plasma $C_{max\ (0\text{-}12\ h)}$[4], nmol/L	1.95	5.06	0.30	<0.001
Plasma $T_{max\ (0\text{-}12\ h}$[5]), h	4.44	4.31	0.88	<0.001
0-h plasma EL, nmol/L	1.10	1.42	0.18	0.22
12-h plasma EL, nmol/L	1.16	3.21	0.30	<0.001
24-h plasma EL, nmol/L	1.07	2.67	0.19	<0.001
48-h plasma EL, nmol/L	1.07	2.05	0.28	<0.001
$AEA_{0\text{-}12\ h}$[6], %	1.85	1.31	0.20	0.08

[1]Plasma EL data reported in this table were obtained from d 5 to d 7 of life.
[2]Significance was declared at $P \leq 0.05$ and trends at $0.05 < P \leq 0.10$.
[3]Area under the curve between 0 and 12 h ($AUC0_{\text{-}12\ h}$) on d 5 of file was calculated by subtracting baseline values (0-h plasma EL) from all remaining time points according to the trapezoidal rule (Phillips and Taylor, 1973).
[4]Maximum plasma concentration of EL between 0 and 12 h [$C_{max\ (0\text{-}12h)}$] on d 5 of life.
[5]Time to reach C_{max} between 0 and 12 h [$T_{max\ (0\text{-}12\ h)}$] on d 5 of life.
[6]Apparent efficiency of absorption between 0 and 12 h ($AEA_{0\text{-}12\ h}$); $AEA_{0\text{-}12\ h}$, % = [plasma EL (mg/L) × BW (kg) × 0.092 ÷ EL intake (mg)] × 100 (Quigley et al., 1998).

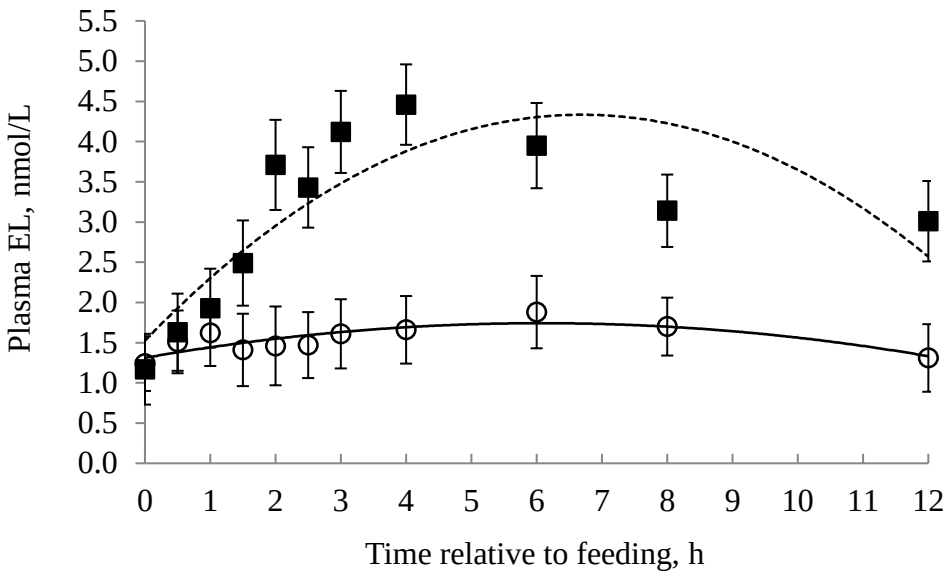

Figure 7. Plasma concentration time curve of enterolactone (EL) after oral administration of 2 L of milk replacer (Low-EL treatment; ○) or 2 L of EL-enriched milk (High-EL treatment; ■) to newborn calves on d 5 of life. Values are means ± SD.

APPENDIX I: REQUIRED DOCUMENTS FROM IACUC

IACUC no. 140901 from chapter II:

University *of* New Hampshire

Research Integrity Services, Service Building
51 College Road, Durham, NH 03824-3585
Fax: 603-862-3564

19-Sep-2014

Brito, Andre Fonseca De
UNH Farms, Dairy T & R Ctr
Durham, NH 03824

IACUC #: 140901
Project: Effects of Supplementing Different Levels of Liquid Molasses on Animal Production, Milk Enterolactone Concentration, and Nutrient Utilization in Organic Jersey Cows Fed Flaxseed Meal
Category: C
Approval Date: 18-Sep-2014

The Institutional Animal Care and Use Committee (IACUC) reviewed and approved the protocol submitted for this study under Category C on Page 5 of the Application for Review of Vertebrate Animal Use in Research or Instruction - *the research potentially involves minor short-term pain, discomfort or distress which will be treated with appropriate anesthetics/analgesics or other assessments.* The IACUC made the following comment(s) on this protocol:

1. The investigator needs to remove the statement about the surgical procedures form from page 9, and correct the typographical errors on page 6.

Approval is granted for a period of three years from the approval date above. Continued approval throughout the three year period is contingent upon completion of annual reports on the use of animals. At the end of the three year approval period you may submit a new application and request for extension to continue this project. Requests for extension must be filed prior to the expiration of the original approval.

Please Note:
1. All cage, pen, or other animal identification records must include your IACUC # listed above.
2. Use of animals in research and instruction is approved contingent upon participation in the UNH Occupational Health Program for persons handling animals. Participation is mandatory for all principal investigators and their affiliated personnel, employees of the University and students alike. Information about the program, including forms, is available at http://unh.edu/research/occupational-health-program-animal-handlers.

If you have any questions, please contact either Dean Elder at 862-4629 or Julie Simpson at 862-2003.

For the IACUC,

Jill A. McGaughy, Ph.D.
Chair

IACUC no. 160603 from chapter III:

University *of* New Hampshire

Research Integrity Services, Service Building
51 College Road, Durham, NH 03824-3585
Fax: 603-862-3564

16-Jun-2016

Brito, Andre Fonseca De
Biological Sciences
Keener Dairy Research
Durham, NH 03824

IACUC #: 160603
Project: Effects of Feeding Molasses of Flax Oil on Rumen Microbial population and Milk Enterolactone Concentration of Cows Fed Flaxseed Meal
Approval Date: 16-Jun-2016

The Institutional Animal Care and Use Committee (IACUC) reviewed and approved the protocol submitted for this study under Category D on Page 5 of the Application for Review of Vertebrate Animal Use in Research or Instruction - *Animal use activities that involve accompanying pain or distress to the animals for which appropriate anesthetic, analgesic, tranquilizing drugs or other methods for relieving pain or distress are used.*

Approval is granted for a period of three years from the approval date above. Continued approval throughout the three year period is contingent upon completion of annual reports on the use of animals. At the end of the three year approval period you may submit a new application and request for extension to continue this project. Requests for extension must be filed prior to the expiration of the original approval.

Please Note:
1. All cage, pen, or other animal identification records must include your IACUC # listed above.
2. Use of animals in research and instruction is approved contingent upon participation in the UNH Occupational Health Program for persons handling animals. Participation is mandatory for all principal investigators and their affiliated personnel, employees of the University and students alike. Information about the program, including forms, is available at http://unh.edu/research/occupational-health-program-animal-handlers.

If you have any questions, please contact either Dean Elder at 862-4629 or Julie Simpson at 862-2003.

For the IACUC,

Jill A. McGaughy, Ph.D.
Chair

IACUC no. 150303 from chapter IV:

University *of* New Hampshire

Research Integrity Services, Service Building
51 College Road, Durham, NH 03824-3585
Fax: 603-862-3564

07-Apr-2015

Brito, Andre Fonseca De
Biological Sciences, Keener Dairy Research
Durham, NH 03824

IACUC #: 150303
Project: Feeding Calves Enterolactone-Enriched Milk
Category: C
Approval Date: 26-Mar-2015

The Institutional Animal Care and Use Committee (IACUC) reviewed and approved the protocol submitted for this study under Category C on Page 5 of the Application for Review of Vertebrate Animal Use in Research or Instruction - *the research potentially involves minor short-term pain, discomfort or distress which will be treated with appropriate anesthetics/analgesics or other assessments.*

Approval is granted for a period of three years from the approval date above. Continued approval throughout the three year period is contingent upon completion of annual reports on the use of animals. At the end of the three year approval period you may submit a new application and request for extension to continue this project. Requests for extension must be filed prior to the expiration of the original approval.

Please Note:
1. All cage, pen, or other animal identification records must include your IACUC # listed above.
2. Use of animals in research and instruction is approved contingent upon participation in the UNH Occupational Health Program for persons handling animals. Participation is mandatory for all principal investigators and their affiliated personnel, employees of the University and students alike. Information about the program, including forms, is available at http://unh.edu/research/occupational-health-program-animal-handlers.

If you have any questions, please contact either Dean Elder at 862-4629 or Julie Simpson at 862-2003.

For the IACUC,

Jill A. McGaughy, Ph.D.
Chair

IACUC no. 150303 from chapter IV (modification):

University *of* New Hampshire

Research Integrity Services, Service Building
51 College Road, Durham, NH 03824-3585
Fax: 603-862-3564

24-Jun-2015

Brito, Andre Fonseca De
Biological Sciences
Keener Dairy Research
Durham, NH 03824

IACUC #: 150303
Project: Feeding Calves Enterolactone-Enriched Milk
Category: C
Modification Approval Date: 22-Jun-2015
Annual Approval Expiration Date: 26-Mar-2016
Protocol Three-Year Approval Expiration Date: 26-Mar-2018

The Institutional Animal Care and Use Committee (IACUC) has reviewed and approved the requested modification to the protocol for this study:

Changes per June 22, 2015 email

If you have any questions, please contact either Dean Elder at 862-4629 or Julie Simpson at 862-2003.

For the IACUC,

Jill A. McGaughy, Ph.D.
Chair

cc: File

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November 7, 2017

Caren P. Ghedini
Department of Molecular, Cellular, & Biomedical Sciences - MCBS
30' Okane Road
Durham, NH 03824

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Sincerely,

Susan Pollock

Susan Pollock
Managing Editor
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